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Selection of protease inhibitors to prevent or attenuate inflammatory processes

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Selectie van protease inhibitors om ontstekingsprocessen te verhinderen of verminderen
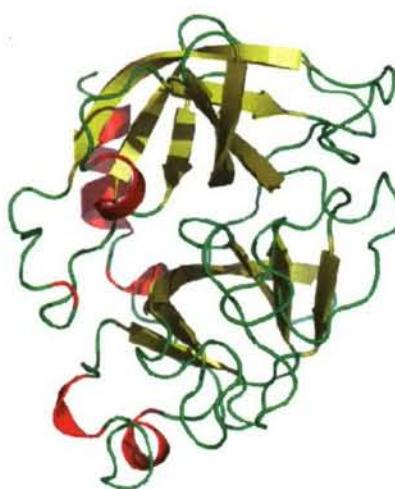
Probleemstelling

Tijdens een infectie wordt weefselschade veelal toegebracht door proteasen afkomstig van het infectieuze agens of van de gastheer. Regulering van humane proteasen is gebaseerd op een nauwe balans tussen pro- en anti-inflammatoire signalen. Wanneer de regulatie van proteasen verstoord is, treden pathologische gebeurtenissen op (bijvoorbeeld microbiële invasie en inflammatoire weefselschade). Deregulering is vaak een onderliggend probleem bij ontstekingsziekten. Sommige bacteriële proteasen kunnen op effectieve wijze humane proteasen activeren waarbij

vervolgens weefselschade wordt veroorzaakt. Daarnaast hebben bacteriële proteasen een functie in het activeren van virulente factoren. Op verzoek van Defensie wordt beoogd om generieke protease-remmers te identificeren en te testen op hun vermogen om weefselschade te voorkomen dan wel te beperken.

Dit onderzoek is uitgevoerd door TNO Defensie en Veiligheid, locatie Rijswijk. Het onderzoek is gefinancierd in het kader van het programma V502 en beantwoordt aan deliverable nummer 7.3.1.

Beschrijving van de werkzaamheden

De werkzaamheden bestonden uit een literatuurstudie waarin gekeken werd welke bacteriële en humane proteasen en protease-remmers een rol spelen bij weefselschade en of deze zijn in te delen in groepen. Naar aanleiding van de resultaten van dit rapport dient een gefundeerde keuze gemaakt te worden welk soort onderzoek uitgevoerd zal gaan worden in het verdere verloop van het project, met als doel een liefst 'generieke' proteaseremmer te ontwikkelen waardoor infectieschade beperkt kan worden.

Resultaten en conclusies

Het selecteren van generieke protease-remmers werd uitgevoerd voor zowel bacteriële als humane proteases.

Met behulp van het MEROPS-programma werd een bacterieel peptidase gevonden waartegen een mogelijk generieke protease-remmer te ontwikkelen is. Het type 4 prepilin peptidase wordt door pathogene bacteriën gebruikt om een pilus te vormen, die verantwoordelijk is voor verschillende virulente factoren (biofilmvorming, uitscheiding van toxines, enzymen, opname van DNA). Uit onderzoek is gebleken dat bij *Francisella tularensis* de virulentie geremd werd wanneer het gen, coderend voor het type IV pilin, gemuteerd werd. Een remmer tegen dit peptidase zou een mogelijk therapeutisch middel zijn om de virulentie van *F. tularensis* en ook andere pathogene bacteriën te bestrijden.

Een nadeel van het selecteren van een bacteriële proteaseremmer is dat infectie-schaden, die veroorzaakt worden door andere BW-agentia (virussen en toxines), niet behandeld kunnen worden. Het zou daarom meer voor de hand liggen om een remmer te selecteren voor humane

proteases die een functie hebben in de
respons die optreedt bij het begin van een
infectie-/ontstekingsreactie. Het kallikreïne-
kininesysteem biedt de mogelijkheid om in
te grijpen in de gastheerrespons zodat
bescherming geboden kan worden tegen
infectieschade. Door remming van kinine-

receptor B_1 en stimulatie van kininereceptor
B_2 (in combinatie met de remming van het
angiontensin converting enzyme), zou een
mogelijke therapeutische behandeling
ontwikkeld kunnen worden waardoor de
gastheer beschermd wordt tegen infectie-
schade.

PROGRAMMA

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Programmatitel
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Met de programma/project-
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PROJECT

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Projecttitel
Beperking infectieschade

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Samenvatting

Het ontstekingsproces is een gecoördineerde respons om de mens te beschermen nadat een infectie is opgetreden. Humane proteasen spelen een belangrijke rol in dit proces. Serineproteasen van het complement systeem spelen een directe rol bij het bestrijden van binnengedrongen micro-organismen terwijl matrix metalloproteasen (MMP's) kritische mediatoren zijn voor weefselremodelling. De regulering van deze proteasen wordt uitgevoerd door een balans tussen pro- en anti-inflammatoire signalen. Wanneer de regulatie van bijvoorbeeld MMP's verstoord is, treden er vele pathologische gebeurtenissen op, bijvoorbeeld microbiële invasie en inflammatoire weefselschade.

Ook bacteriële proteasen kunnen de regulatie van humane proteasen beïnvloeden waarbij de balans tussen pro- en anti-inflammatoire signalen verstoord wordt. Bacteriële proteasen kunnen ook een directe uitwerking hebben door het afbreken van weefselbestandelen of antilichamen. Het doel van dit onderzoek is om in een literatuurstudie te kijken welke bacteriële en humane proteasen een rol spelen bij weefselschade en hier op volgend een generieke proteaseremmers te identificeren op hun vermogen om weefselschade te voorkomen dan wel te beperken.

Het ontwikkelen van proteaseremmers tegen MMP's is een complexe aangelegenheid. De activiteit van MMP's wordt gereguleerd op diverse niveaus en elk MMP wordt geproduceerd en geactiveerd in een cascade van reacties die ook door remstoffen beïnvloed kunnen worden. Uit onderzoek is gebleken dat wanneer het ene MMP geremd wordt, de activiteit overgenomen kan worden door een ander MMP. Ook bleek in sommige gevallen dat remming van een MMP de ontstekingsreactie versterkte in plaats van te verzachten. Ingrijpen in deze processen is daarom niet mogelijk tot het moment aangebroken is waarop de organisatie en regulatie van MMP's totaal duidelijk is. Een dergelijke situatie geldt ook voor caspases, die betrokken zijn bij productie van cytokines en een rol spelen bij apoptose, en proteasen in het complement systeem. Wanneer bacteriën het lichaam binnendringen, hebben ze voordeel wanneer voedingsstoffen worden aangeleverd. Bradykinine vergroot de permeabiliteit van bloedvaatwanden door bradykinine-2 receptoractivatie in het kallikreïne/kinine systeem en speelt een rol in ontstekingsprocessen. Door modulatie van het kallikreïne/kinine systeem zijn bacteriën hierdoor in staat om extra voedingsstoffen te krijgen voor groei en proliferatie. Het ligt dan voor de hand om dit te voorkomen door de synthese van bradykinine te verhinderen of de B_2-receptor te blokkeren met een bradykinine antagonist. Diverse onderzoeken hebben echter aangetoond dat voor de bestrijding van een bacteriële infectie, een werkend kallikreïne/kinine systeem noodzakelijk is en een onderdeel uitmaakt van processen waarbij bacteriën uit het lichaam verwijderd worden. In een infectiemodel werd aangetoond dat het type 1 immuniteit versterkt werd door bradykinine, door een samenspel van Toll-like receptor 2 (TLR2), de bradykine-2 receptor en angiotensin-omzettend enzym.

Met behulp van het MEROPS programma werden van de A- en B-lijst NIAID Priority Pathogens, peptidasen geselecteerd, waartegen een generieke proteaseremmer ontwikkeld zou kunnen worden. Uit het databaseonderzoek bleek dat het type 4 prepilin peptidase een protease is waarvan de homologe genoomsequentie voorkomt in het merendeel van de pathogenen die beschreven staan in de A- en B-list NIAID Priority Pathogens. Het type 4 prepilin peptidase wordt door pathogene bacteriën gebruikt om een pilus te ontwikkelen, die verantwoordelijk is voor verschillende virulente factoren. Uit onderzoek is gebleken dat de virulentie van *Francisella tularensis* geremd werd wanneer het gen, coderend voor het type IV pilin, gemuteerd werd. Een remmer tegen

dit peptidase zou een mogelijk therapeutisch middel zijn om de virulentie van
F. *tularensis* en ook van andere pathogene bacteriën te bestrijden. Proteaseremmers
kunnen ontwikkeld worden via *in vitro* evolution of door het selecteren van
synthetische remstoffen tegen het peptidase.

Een nadeel van het selecteren van een bacteriële proteaseremmer is dat infectieschade,
die veroorzaakt worden door andere BW-agentia (virussen en toxines), niet behandeld
kunnen worden. Het zou daarom meer voor de hand liggen om een remmer te selecteren
voor humane proteases die een functie hebben in de respons die optreedt bij het begin
van een infectie-, ontstekingsreactie. Het kallikreïne-kinine systeem biedt de
mogelijkheid om in te grijpen in de gastheerrespons zodat bescherming geboden wordt
tegen infectieschade. Door remming van kininereceptoren en dit te combineren met het
ingrijpen in andere immunologische reacties, gerelateerd aan het bradykinine/kinin
systeem, zou een mogelijke therapeutische behandeling ontwikkeld kunnen worden
waardoor de gastheer beschermd wordt tegen infectieschade.

Summary

Inflammation is a coordinated response aimed at the protection of the host at the onset of infection. In this process, human proteases play a critical role. Serine proteases of the complement system attack bacteria by the formation of a membrane attack complex. Matrix metalloproteases are critical mediators in tissue remodeling. The regulation of these proteases is carried out by pro- and anti-inflammatory signals. Deregulation of human (metallo)peptidases results in many pathological reactions such as microbial invasion or inflammatory tissue damage. Also bacterial proteases can influence the balance between pro- and anti-inflammatory signals and thus cause tissue damage. Bacterial proteases can also directly act as infectious agents, for example the breakdown of tissue components. The aim of this research is to address the microbial and human interactions which results in inflammation reactions. As a result generic protease inhibitors have to be identified that can prevent or attenuate tissue damaging processes. The development of protease inhibitors against MMPs is very complex. MMPs are entwined in a complex cascade with themselves and other proteases and these proteases could try to compensate if an MMP is blocked. The complex cascade is also controlled by endogenous inhibitors. It has been shown that MMP inhibition actually can stimulate disease progression, possibly by inhibiting beneficial proteases. Synthesis of protease inhibitors in this field is therefore hardly possible, until the organisation and regulation of MMPs is totally clear. A similar cascade of (in)activation reactions takes places in the regulation of the activity of caspases and proteases involved in the complement system.

Bacteria can use the kallikrein/kinin system for the acquisition of plasma proteins (for growth and proliferation) by deregulating this system which results in an overproduction of bradykinin, a hormone that increases capillary permeability.

To reduce the effects of bradykinine, the kallikrein/kinin system can be modulated. However, several research groups have recently shown that a working kallikrein/kinin system is needed for bacterial clearance. In an infection model the type 1 immunity was vigorously induced by bradykinine, an innate signal whose levels in peripheral tissues are controlled by an intricate interplay of Toll-like receptor 2, bradykinine-2 receptor and angiontensin converting enzyme. The kallikrein/kinin system can be used for the development of novel therapies for treatment of bacterial infections.

To find generic bacterial peptidases that could possibly act as target for medical counter measures, the MEROPS database was used. For the A- and B-list NIAID Priority Pathogens, the type IV prepilin peptidase was found to be a peptidase for which a generic peptidase inhibitor could be developed. Type IV prepilins are required for functions including type IV pilus formation, toxin and other enzyme secretion, gene transfer and biofilm formation. Therefore type IV prepilins are important virulence factors. The pilins can perform these functions only after processing by a prepilin peptidase. Recently, research showed that the virulence of *Francisella tularensis* was attenuated when the type IV pilin gene was mutated. A protease inhibitor against the type IV prepilin peptidase could therefore be of therapeutical value. These inhibitors can be developed by *in vitro* evolution or by selection of synthetic inhibitors.

A disadvantage of selecting a bacterial protease inhibitor is that inflammation caused by other biowarfare agents like viruses or toxins can not be treated. It would be more appropriate to select human proteases which have a function in the coordinated response aimed at the protection of the host at the onset of an inflammatory response. The kallikrein-kinin system can modulate both the innate and adaptive immunity and could therefore represent a promising approach for the development of novel strategies to treat bacterial infections. Efforts in future research in which the blockade of kinin receptors only or in combination with other compounds might result in the development of treatment to protect the host at the onset of infection.

Contents

Appendices

Abbreviations

α1-ACT	antichymotrypsin
α1-MG	macroglobulin
α1-PI	α2-proteinase inhibitor
α2-MG	α2-macroglobulin
ACE	Angiotension converting enzyme
ADAM	a disintigrin and a metalloproteinase domain
Ag	aggregan
Amg	amelogenion
ANG	angiotensin
Apaf-1	apoptotic protease activating factor 1
APC	activated protein C
BIR	baculoviral IAP repeat
BK	bradykinin
BoNT	botulinum neurotoxin
B_1R	kinin receptor B_1
B_2R	kinin receptor B_2
Cas	casein
CARD	caspase recruitment domain
CD antigen	differentiation antigens (markers) of lymphocyte subsets on cell membrane which are defined by monoclonal antibodies
CD11	Leukocyte adhesion molecule : receptors on leukocytes or target tissues that mediate leukocyte\- target cell adhesion and, in conjunction with antigen binding to the T cell receptor, lymphocyte activation
CNF	congenital nephritic syndrome
Coll	collagen
CpG	unmethylated CpG dinucleotides in particular base contexts (CpG motifs)
CTGF	Connective Tissue Growth Factor
CZP	cysteine protease cruzipain
Dc	decorin
DFP	Di-Isopropyl Fluorophosphate
DISC	Death inducing signaling complex
DNA	deoxyribonucleic acid
dsRNA	dubble stranded RNA
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
EGTA	Acetic acid, (ethylenebis(oxyethylenenitrilo)) tetra-; ethylene glycol bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
El	elastin
En	entactin
FADD	Fas associated death domain protein
Fb	fibrin/fibrinogen
FGF-R	fibroblast growth factor receptor
Fn	fibronectin
Gel	gelatin
HAE	hereditary angiodema
HB-EGF	heparin binding epithelial growth factor-like growth factor
HMWK	high molecular weight kininogen

HOCl	hypochlorous acid
HUS	Hemolytic uremic syndrome
IAP	inhibitor of apoptosis
IBD	inflammatory bowel disease
ICAM	intercellular cell adhesion molecule
IgA1	Immunoglobulin A1
IGF-BP	insulin-like growth factor binding protein
IKKI	inducible I-kappa-B kinase
IL	interleukin
IL-1β	interleukin-1β
IMPI	Inhibitor of Metalloproteinases from Insects
iNOS	inducible nitric oxide synthase
Kgp	Lys-specific cysteine proteinase (Lys-gingipain)
LBK	Lys-bradykinin, kallidin
LFA	lymphocyte function associated antigen
LIF	leukemia inhibitory factor
Lm	laminin
LMWK	low molecular weight kininogen
LPS	lipopolysaccharide
LT	heat-labile enterotoxin
LTA	lipoteichoic acid
LVS	live Vaccine Strain
MAC	membrane attack complex
MALP-2	macrophage-activating lipopeptide 2
MAPKK	mitogen-activated protein kinase kinase
MBL	mannan-binding lectin
MCP	monocyte chemoattractant protein
MD-2	an accessory protein of the TLR-4
MEROPS	The idea of using the terms 'family' and 'clan' for the groups of peptidases came from a television documentary on bee-eaters, and because of this, the generic name of the bee-eater was chosen as the name of the database. In Greek mythology, Merops was a Trojan seer who was father-in-law to Priam, the King of Troy. The name was applied by Linnaeus to the European Bee-eater (*Merops apiaster*) in 1758.
MHC	major histocompatibility complex
MMP	matrix metalloproteases
MT-MMP	membrane type MMP
MurNac	N-acetylmuramic acids
NEP	neutral endopeptidase
NF-κB	nuclear factor κB
NIAID	National Institute of Allergy and Infectious Diseases
NLR	NOD-like receptors
ON	osteonectin
PAMPs	Pathogen associated molecular patterns
PAR	protease-activated receptors
PDGF	platelet derived growth factor
Per	perlecan
PF	platelet factor
Pgl	proteoglycans
PGN	Peptidoglycan
PIDD	p53 induced death domain protein

Pl	plasminogen
PMN	polymorphonuclear neutrophils
PMSF	Phenylmethanesulfonyl Fluoride
PRR	Pattern Recognition Receptors
RA	rheumatoid arthritis
RgpB	arginine-specific gingipains-R
rhAPC	recombinant human activated protein C
RNA	ribonucleic acid
SF	scarlet fever
SSS	Staphylococcal Scalded Skin Syndrome
ST	heat stable enterotoxin
TACE	TNF-α converting enzyme
TCR-α	T-cell receptor α
TEM	Transmission Electron Microscopy
Tfp	type IV pili
TGF	transforming growth factor
TGF-β	transforming growth factor β
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	toll-like receptors
Tn	tenascin
TNF- α	tumor necrosis factor α
TRAF	TNF receptor-associated factor
TSS	toxic shock syndrome
tTG	tissue transglutaminase
Umu	UV mutagenesis
UTI	urinary tract infection
VAMP	vesicle associated membrane protein
VCAM	vascular cell adhesion molecule
Vn	vitronectin

1   Introduction

1.1   Background

Proteolytic enzymes play several physiological roles and are essential factors for homeostatic control in both prokaryotes and eukaryotes. Microbial proteases have physiological roles in the life cycle of these organisms but act occasionally as toxic factors to the host. Metallo-, cysteine- and serineproteases are widely spread in many pathogenic bacteria and play a critical role related to colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection.

Inflammation is a coordinated response aimed at the protection of the host at the onset of infection. Human peptidases, especially the matrix metallopeptidases, are critical mediators in tissue remodelling. Deregulation of the human peptidases results in many pathological reactions such as microbial invasion or inflammatory tissue damage. Most often deregulation of inflammation is caused by bacterial peptidases that effectively activate host pro-peptidases by degrading the auto inhibitory domains of these enzymes. Since these activated host peptidases are able to degrade host tissue or increase inflammation processes, effective therapeutic treatment should be aimed to inhibit the activation of (metallo)peptidases. The development of bacterial protease inhibitors can result in effective therapeutic treatment, because resistance to pharmacological agents (antibiotics, which act as inhibitors of bacterial cell wall biosynthesis or affecting protein synthesis on ribosomes) is a serious medical problem.

1.2   Goal of the literature review

Bacterial and human proteases play a significant role in inflammation reactions. The aim of this literature research is to select human and/or bacterial peptidases that could act as a target for generic peptidase inhibitors. Subsequently, inhibitors have to be synthesized and their ability to inhibit proteases should be tested *in vitro* and *in vivo* experiments in order to reduce tissue damage. This review fits in with the program V502, MTM-B: project number 7.3, entitled 'Limitation of tissue damage'.

This review sets out to address the microbial and human interactions which results in inflammation reactions. In Chapter 2, the process of tissue inflammation is described, especially the innate immune response responsible for the initial defense against infection. In Chapter 3 an overview of human and bacterial proteases involved in inflammation is given. A detailed description of human proteases and the processes in which they are involved show the complexity of the human immune system. To select bacterial proteases for which generic inhibitors can be synthesized, the MEROPS database was used. This selection is described in Chapter 4. Chapter 5 gives an overview of protease inhibitors and the possibility to select and generate synthetic inhibitors will be discussed. Conclusions and recommendations for future work are given in Chapters 6 and 7. The choice for the bacterial prepilin peptidase or the human kallikrein-kinin system will be explained.

2   Process of tissue inflammation

2.1   Inflammation

Inflammation is the first response of the immune system to infection or irradiation and may be referred to as the innate cascade. In the first phase of inflammation, blood vessels are dilated upstream of an infection and constricted downstream. The capillary permeability to the injured tissue is increased, resulting in a loss of blood plasma into the tissue. These events cause redness and heat and give rise to edema or swelling. The plasma which is entering the injured tissue contains many components and cells. The ligamentous and cellular debris and a number of chemicals in the plasma around damaged cells attract an influx of white blood cells called leukocytes (neutrophils). Neutrophils take on an important role in inflammation. Their job is to clean out bacteria and prevent infection at the injured site. Granula of neutrophils contain several components to attack bacteria. The content of neutrophilic granula is described in Appendix A. Neutrophils also secrete hormones which attract macrophages. The arrival of the macrophages signals the beginning of the next phase, the granulation phase. The macrophages clean up the area by digesting the degraded cell parts and secreting enzymes (matrix metalloproteases [MMP]), which break down many of the damaged ligament molecules. They release TNF-α and IL-1 in response to activation of toll-like receptors. The macrophages also secrete growth factors which stimulates the growth of new blood vessels and intercellular matrix. When fibroblasts are turned on by macrophages, they rapidly make massive amounts of building blocks of the extracellular matrix (ECM, collagen, elastine and proteoglycans). For the remodelling of tissue they also produce MMPs and TIMPs (Tissue Inhibitors of MetalloProteases). If inflammation of the affected site persists, released cytokines IL-1 and TNF-α will activate endothelial cells to upregulate receptors VCAM-1, ICAM-1, E- and L-selectin for various immune cells. This receptor upregulation increases extravasation of neutrophils, monocytes, activated T-helper and T-cytotoxic, and memory T and B cells to the infected site.

2.2   The innate immune response

When bacteria enter the human body, the immune system responds through a diverse set of mechanisms in an attempt to eliminate the infectious agent. These immune responses can be segregated into two compartments, namely the innate (nonspecific) and the adaptive (specific) immune systems. The adaptive immune response is made up of B and T lymphocytes that have unique receptors specific to various microbial antigens. In the cellular immune response, T-lymphocytes kill cells that display foreign motifs on their surface. They also stimulate the humoral immune response by helping B cells, the precursors of plasma cells. The recognition molecules of the humoral immune response are soluble proteins called antibodies. Although the size and diversity of the lymphocyte repertoire make it likely that there is a specific lymphocyte for any given antigen, the frequency of these cells can be extremely low and normally will not be sufficient to protect the host against a primary infection. Therefore only the innate immune response will be discussed in more detail which is involved in the initial defense against infection of which inflammation is an important part. The term, innate immunity, refers to the basic resistance to disease that an organism possesses - the first

line of defense against infection. The characteristics of the innate immune response include the following:

- responses are broad-spectrum (non-specific);
- there is no memory or lasting protective immunity;
- there is a limited repertoire of recognition molecules;
- the responses are phylogenetically ancient.

The elements of the innate immune response are:

- anatomic barriers (skin, epidermis, mucous membranes);
- physiologic barriers (temperature, elevated body temperature, pH, oxygen tension);
- number of chemical factors (fatty acids, lactid acid, pepsin, lysozyme, antimicrobial peptides, interferons, complement);
- endocytic and phagocytic barriers endocytosis (process by which macromolecules contained within the extracellular tissue fluid or whole pathogenic microorganisms are internalized by cells. Phagocytosis is carried out by the so-called 'professional phagocytes' (monocytes and macrophages, neutrophils, and dendritic cells)).

Because this study is focusing on the microbial and human interactions which results in inflammation reactions, the complement system and the immediate recognition of antigenic structures common to many microbes resulting in an inflammatory response, are described in more detail.

2.2.1   *Complement system*

The complement system organizes and attack on bacterial invaders in order to clear pathogens from an organism. It is composed of nine plasma proteins designated C1 to C9 and several fragments indicated by lower-case letters. The complement system can be activated in response to the recognition of targets. Recognition occurs by three pathways:

- the classical pathway;
- the lectin (or MBLectin) pathway;
- the alternative pathway.

Activation of this system leads to cytolysis, chemotaxis, opsonisation, immune clearance, inflammation as well as the marking of pathogens for phagocytosis.
The action of the complement system affects both innate immunity and acquired immunity [Sim and Laich, 2000].

2.2.2   *PAMPs*

The innate response relies on immediate recognition of antigenic structures common to many microbes and that are typically absent in host systems. These highly conserved microbial structures are termed Pathogen Associated Molecular Patterns (PAMPs). Well-described PAMPS include the following molecules.

- LPS; Lipopolysaccharides or endotoxin, is ubiquitously expressed on the outer membrane of gram-negative bacteria [Kengatharan et al., 1998].
- LTA; Lipoteichoic acids a compound of the cell wall of gram-positive bacteria [Wicken and Knox, 1975].
- Peptidoglycan; provides shape and mechanical rigidity to bacteria. Peptidoglycan is composed of disaccharides of N-acetylglucosamine and N-acetylmuramic acids (MurNAc) linked to pentapeptides [Green, 2002].

- Flagella; Flagellum, the motor organelle that facilitates bacterial movement consists of the basal body, flagellar motor, switch, hook, flagellar filament, capping proteins, junction protein and export apparatus [Macnab, 2003].
- Bacterial DNA and viral double stranded RNA; two features distinguish bacterial DNA from mammalian DNA: an abundance of CpG dinucleotides and the lack of cystosine methylation in these sequences [Bird et al., 1987].

Recognition of PAMPs is based on the interactions of these molecules with Pattern Recognition Receptors (PRRs), such as toll-like receptors (TLRs), which are expressed on various cells of the innate immune response including dendritic cells and macrophages [Medzhitov et al., 1997]. The following TLRs have been found to bind several ligands (Table 1).

Table 1   Toll-like receptors and their ligands.

Toll-like receptor	Ligand
TLR2/1	?
TLR2/6	PGN, MALP-2, Zymosan
TLR2/?	LPS *Leptospira* LPS *P. Gingivalis* Lipoprotein Lipoarabinomannan
TLR3	dsRNA, Poly (I:C)
TLR4	LPS
TLR5	Flagellin
TLR7	Imidazoquinolines
TLR8	Single stranded RNA
TLR9	CpG DNA
TLR10	?

2.2.3   *Activation of the inflammatory cascade signal transduction pathways*
When bacterial products/compounds bind to diverse receptors (TLR), the signalling
pathways typically converge on nuclear factor (NF-κB, Figure 1). NF-κB is a key player
in the activation of inflammation, and suppression of apoptosis [Jobin and Sartor,
2000]. NF-κB controls the expression of essentially all pro-inflammatory cytokines,
chemokines, immune receptors, and cell surface adhesion molecules (IL-1β, TNF-α,
IL-6, -8 and −12, iNOS, ICAM-1, VCAM-1, TCR-α and MHC class II molecules).
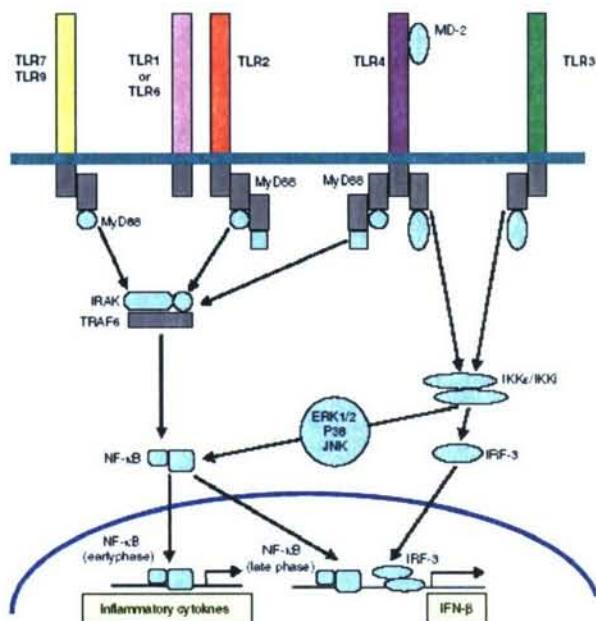
Figure 1   Toll-like receptor signaling pathways [Madianos et al., 2005]. LPS, which activates TLR-4
bind first to lipopolysaccharide binding protein (LBP) and is then transported to CD14. CD14
is a soluble or membrane bound protein that transfers the complex to the receptor. TLR4 also
needs another protein MD-2 to be activated. Once activated, TLR4 recruit myeloid
differentiation factor 88 (MyD88), which associates with serine-threonine protein kinase IL-1
receptor-associated kinase (IRAK) and TNF receptor-associated factor-6 (TRAF-6) adaptor
protein. Oligomerization of TRAF-6 activates a group of MAPK kinase kinases, which directly
or indirectly leads to activation of IκB kinase 1 (IKK1) and IKK2. These kinases phosphorylate
IκB on serine residues thus targeting IκB for degradation and releasing nuclear factor-κB
(NF- κB), which translocates to the nucleus and induces de novo synthesis of inflammatory and
immune response genes [Madionos et al., 2005).

2.3   Apoptosis as a proinflammatory event

Infection of cells by some pathogenic bacteria triggers host cell apoptosis
[Zychlinsky and Sansonetti, 1997]. Apoptosis, or programmed cell death, is the
expression of specific surface receptors that allow phagocytes to recognize and engulf
cellular cadavers, thus avoiding spillage of intracellular contents that could cause tissue
destruction and inflammation. A unique (human) family of cysteine proteases, caspases,
execute programmed cell death. These enzymes will be discussed in Paragraph 3.2.6.
Bacteria-induced apoptosis appears to promote an inflammary response that causes
tissue damage and further bacterial colonization. Bacterial infections where apoptosis
may play a pivotal role in inflammation are: salmonellosis; diphteria; lethal shock by
anthrax toxin; listeriosis and shigellosis. Salmonella induced apoptosis in macrophages
is mediated by large vacuole formation. Diphteria toxin induces macrophage apoptosis

by binding to elongation factor 2 and inhibiting translation. The lethal factor LeTx from *Bacillus anthracis* is a particularly interesting example: this zinc-dependent protease cleaves mitogen-activated proteinase kinase kinase leading to apoptotic cell death of murine macrophage cell lines and human peripheral blood mononuclear cells [Popov et al., 2002]. The toxic shock provoked by the lethal anthrax toxin and shigellosis is mediated by macrophage release of IL-1.

Photobacterium damselae subsp. *piscicida* is an extracellular pathogen which produces an apoptosis inducing protein, AIP56, which induces apoptosis in sea bass macrophages and neutrophils [Vale et al., 2005].

During bacterial-induced apoptosis of neutrophils and macrophages not only cytokines but also reactive oxygen components are released. Hypochlorous acid molecules (HOCl) produced by these cells work synergistically with neutophilic proteases. Protease inhibitors will be inactivated and MMPs will be activated by reactive oxygen compounds [Roos, 1995].
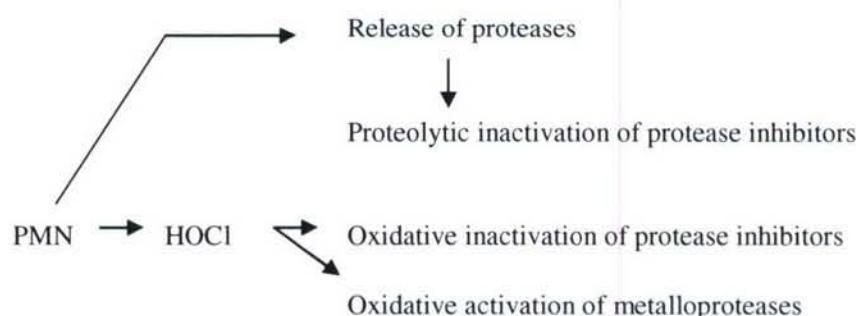
Figure 2   Schematic presentation of synergistically relation between neutrophilic proteases and reactive oxygen compound HOCl (PMN; polymorphonuclear neutrophils).

Several pathogens, including herpes, pox, and baculoviridae have evolved genes that specifically inhibit host cell apoptosis machinery [Spriggs, 1996]. Parasitic pathogens like *Leishmania donovani* seem also to inhibit apoptosis [Moore and Matlashewski, 1994].

3   Bacterial and human proteases involved in inflammation processes

3.1   Proteases

In Chapter 2 the inflammation process is described. In order to find out if generic proteases inhibitors can be selected and synthesized, an overview of human and bacterial proteases involved in the inflammation reaction was made. A description of the function of the proteases will be given.

3.1.1   *Definition of proteases*

Proteases (also termed peptidases, proteinases and proteolytic enzymes) are enzymes that are capable of hydrolyzing peptide bonds of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes is used and are thus classified as hydrolases. There are six classes of proteases based on catalytic types (Table 2). In the MEROPS database, a hierarchical, structure-based classification is used, which is based on that of Rawlings and Barrett (1993), in which the catalytic type of the protein represents the top level in the hierarchical classification. According to this rule, the peptidases can be divided into clans based on three-dimensional protein folding and into families based on evolutionary relationships of the primary sequence. Each clan is identified with two letters, the first representing the catalytic type of the families included in the clan. Some clans are divided into subclans because there is evidence of a very ancient divergence within the clan, for example MA(E), the gluzincins, and MA(M), the metzincins.

Each family is identified by a letter representing the catalytic type of the peptidases it contains together with a unique number for example: Peptidase family A24 (type IV prepilin peptidase family).

Tabel 2   Classes of proteases base don catalytic types.

Protease	Primary role in catalysis	Abbreviation letter
Serine proteases	Amino acid residue, serine	S
Threonine proteases	Amino acid residue, threonine	T
Cysteine proteases	Amino acid residue, cysteine	C
Aspartic acid proteases	Aspartic groups	A
Metalloproteases	Metal ions	M
Glutamic proteases	Glutamic groups	G

The clan of peptidases of unknown catalytic type is described with the abbreviation U. The clan of mixed (C, S, T) catalytic type is described with the abbreviation P.

Serine proteases and matrix metalloproteases (MMPs) are optimally active at neutral pH (neutral proteases) and therefore play the major role in extracellular proteolysis. Most cysteine and aspartic proteinases are optimally active at acidic pH and their main role is in intracellular degradation of proteins in the acidic environment of lysosomes [Owen, C., 2005].

Polymorphonuclear neutrophils (PMNs) in the human body do not synthesize proteinases de novo. Serine proteinases and MMPs are produced in PMN and monocyte precursors in the bone marrow. They are stored in various granules of PMNs and are released from degranulating PMNs [Owen and Campbell, 1999]. Cysteine proteinases

are stored as processed within the lysosomes of many cells. MMPs are generally synthesized and secreted by macrophages. Monocytes have limited capacity to synthesize and secrete MMPs, producing predominantly MMP-7 when they are activated. As monocytes mature into macrophages, they lose their complement of serine proteinases, but develop the capacity to secret MMP-1, -3, -9, -12 and -14. Unlike serine proteinases, which are stored as active enzymes within cells, MMPs are released as proenzymes and are activated within the extracellular space. Activation of most pro-MMPs occurs after their secretion into the extracellular space, which likely is mediated by proteinases and oxidants. Subset of MMPs includes membrane-associated proteinases (membrane type MMPs, MT-MMPs). They are anchored to the cell surface by either a transmembrane domain and they are expressed on the surface of inflammatory cells. ADAMs are a family of 29 cell surface proteinases and are so called because they contain *a d*isintegrin and *a m*etalloproteinase domain. They may contribute to extracellular proteolysis, and may regulate cell adhesion and migration, inflammation, apoptosis and cell signaling [Primakoff and Myles, 2000].

3.2 Human proteases involved in inflammation processes

In this paragraph, human proteases will be discussed which are involved in inflammation processes:
- matrix metalloproteases;
- serine proteases in the complement system;
- protease-activated receptors;
- thrombin;
- kallikrein/kinin system;
- caspases.

3.2.1 Families of metalloproteases

Zinc-containing metalloproteases are widely distributed from prokaryotes to eukaryotes and are classified into four groups, based on the sequence around the zinc binding residues. The group possessing the HEXXH zinc binding motif is called the 'zincins superfamily [Hooper, 1994] (Figure 3). This family is further subdivided into at least ten families on the basis of the third zinc ligand. Degradation of extracellular matrix (ECM) and basement membrane components is an important feature of development, morphogenesis, tissue repair and remodeling. Various types of proteinases are implicated in ECM degradation, but the major enzymes are considered to be matrix metalloproteinases, also called matrixins. They were first described in vertebrates, but have also been found in invertebrates and plants. They are distinguished from other endopeptidases by their dependence on metal ions as cofactors.

Zinc metalloproteases

1 3	1 4	1 5	1 5	- → first and second ligands
HXH	*HXXE*	*HEXXH*	*HXXEH*	
DD-Carboxy-Peptidase	Carboxy-peptidase	Zincins	Inverzincins	

25	40	11	→ third ligand
GXXN*E*XXSD	XE*E*X	XXGXXH	
Thermolysin family	Neurotoxin family	Metzincins	

39 41	56 58	24	19	– – → putative fifth ligand
SXMSY	SXMHY	CIMXP	AXMYP	
Serralysin family	Astacin family	Reprolysin family	Matrixin family	

Figure 3   Families of zinc metalloproteases based on the sequence around the zinc-binding residues. Italicized letters represents identified zinc ligands. Underlined letter represents putative zinc ligand; X stands for any amino acid.

All MMPs are produced as inactive proenzymes, and most of them are secreted to the extracellular environment as latent zymogen. MMPs are classified as the matrixin subfamily of zinc metalloprotease family (Figure 4). Twenty-four different vertebrate MMPs have been identified, 23 of which have been found in humans [Visse and Nagase, 2003]. MMPs are genetically distinct but structurally related. A typical MMP consists of a propeptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable length (hinge region) and a hemopexin (Hpx) domain of about 200 amino acids. They all have the zinc-binding motif in the catalytic domain, and nearly all of them have the cysteine switch motif PRCGXPD in the propeptide maintaining the latent zymogen form. On the basis of specificity, sequence similarity and domain organization, MMPs are divided into six groups: collagenases, gelatinases, stromelysisn, matrilysins, membrane-type MMPs and other MMPs. Collagenases (MMP-1, -8, -13 and-18) are capable of degrading triple-helical fibrillar collagens into ¾ and ¼ fragments. Collagens are the major components of bone and cartilage. Stromelysins (MMP-3, -10 and -11) display a broad ability to cleave extracellular matrix proteins but are unable to use collagens as substrate. Gelatinases cleave type IV collagen and gelatin. Two members of this sub-group are MMP-2 (expressed in most tissues) and MMP-9 (found in neutrophils). Matrilysins (MMP-7 and MMP-26) are the smallest MMPs, they are characterized by the lack of hemopexin domain. In addition to the ECM components digested by them (elastin is the most important subtrate), MMP-7 can also process cell surface molecules. Six membrane-type MMPs (MT-MMPs) have been characterized (MMP-14, -15, -16, -17, -24 and -25). Because the MT-MMPs are membrane bound, they provide a focalized area for ECM proteolytic degradation. With the exception of MT4-MMP, they all have a broad spectrum of substrate specificity, and they are all capable of activating proMMP-2. For their pericellular fibrinolytic activity, MT-MMPs have an important role in skeletal development as well as in angiogenesis [Sternlicht and Werb, 2001]. There are other MMPs not classified in the above-mentioned categories. For example, MMP-12 is mainly expressed in macrophages and is essential for macrophage migration

[Shapiro et al. 1993]. In Appendix B, a detailed list of MMPs and their substrate is shown. In Appendix C, a list of biological activities mediated by MMP cleavage is shown.

During inflammation, inflammatory cells and fibroblasts are stimulated to produce MMP-1, -2, -3 and -9. Biopsies of chronic pressure ulcers has also shown that, compared with normal skin tissue, MMP levels are highly elevated in pressure ulcers and that there is a strong association with inflammatory cells [Schultz et al., 2005].

3.2.2 Serine proteases in the complement system

As was mentioned in Paragraph 2.2.1 the complement system can be divided in three pathways all generating homologues variants of the protease C3-convertase [Atkinson and Frank, 2006; Sim and Laich, 2000].

3.2.2.1 Classical pathway

The classical pathway is triggered by activation of the C1-complex (one molecule of C1q, two molecules of C1r and C1s). C1q's binding to antibodies (classes M and G), complexed with antigens or the surface of pathogens. C1q may directly recognize altered phospholipid distribution on apoptotic cells. This binding process results in conformational changes in C1q molecule which leads to the activation of two C1r (serine protease) molecules. C1r cleaves C1s, another serine protease. The modified C1-complex now binds to and splits C2 and C4, producing C2a/C2b and C4a/C4b. The cleavage of C2 and C4 results in the production of C3 convertase C4b2a. C3 is cleaved into C3a and C3b. C3b complexes to become C3bBb3b, which cleaves C5 into C5a and C5b. C5b with C6, C7, C8 and C9 complex to form the membrane attack complex (MAC). MAC inserts into membrane and initiates cell lysis.

3.2.2.2 Lectin pathway

The lectin pathway is homologous to the classical pathway, but with the opsonin, mannan-binding lectin (MBL) instead of C1q. This pathway is activated by the binding of MBL to sugar moieties on the surface of pathogens, which results in cleavage of C2 and C4 to produce the C3 convertase C4b2a and is the same as that in the classical pathway of complement activation. The pathway has both antibody-depent and -independent modes of activation.

3.2.2.3 The alternative pathway

Its activation is triggered by C3 hydrolysis directly on the surface of a pathogen. It does not rely on a pathogen-binding protein like the other pathways. In the alternatieve pathway, the protein C3 is produced in the liver, and is then cleaved into C3a and C3b by enzymes in the blood. If there is no pathogen in the blood, the C3a and C3b protein fragments will be deactivated. However, when there is a nearby pathogen, some of the C3b is bound to the membrane of the pathogen and will bind to factor B. This complex will then be cleaved by factor D into Ba and the alternative pathway C3-convertase, Bb. At this point, MAC is produced in the same way as that in the classical pathway of complement activation.

Both C3a and C5a complement fragments act as specific chemotaxins for leukocytes and in addition may affect other immunocompetent cells, such as microglia [Möller et al., 1997].

3.2.3 Protease-activated receptors

Proteases acting at the surface of cells generate and destroy receptor agonists and activate and inactivate receptors, thereby making a vitally important contribution to signal transduction. Certain serine proteases that derive from circulation (coagulation factors), inflammatory cells (mast cells neutrophil proteases) and from multiple other sources (epithelial cells, bacteria, fungi) can cleave protease-activated receptors (PARs), a family of four G-protein coupled receptors [Ossovskaya and Bunnett, 2004]. In this way, signal transduction can be initiated or terminated (Figure 4).

A Release of soluble ligand
soluble TNFα
TACE   TNFα

B Activation of a precursor
ANG I
ANG II
ACE

C Inactivation of an agonist
SP
fragments
NEP

D Activation of PARs
thrombin
inactive PAR   active PAR

E Disabling of PARs
cathepsin G
inactive PAR   disabled PAR
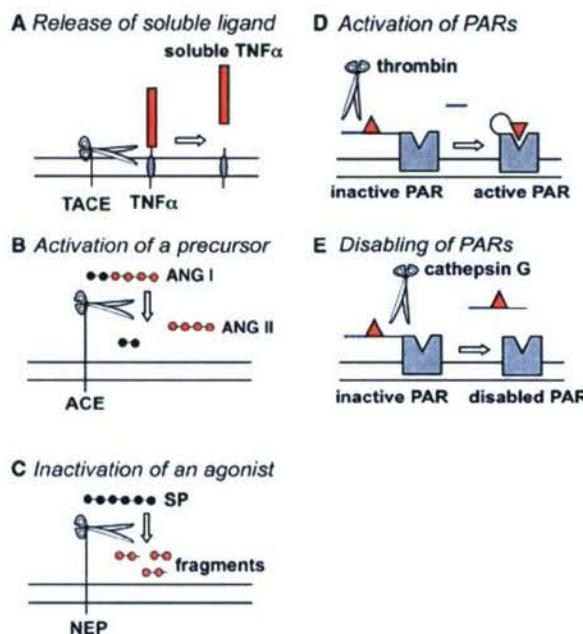
Figure 4   Mechanisms by which cell-surface proteolysis regulates signal transduction. Proteases can regulate signaling by cleaving ligands (A–C) or receptors (D and E) [Ossovskaya and Bunnett, 2004].
A: cell-surface proteases can induce shedding of membrane-bound signaling molecules; e.g., tumor necrosis factor-α (TNF- α)-converting enzyme or TACE, a member of the ADAM family of cell-surface proteases, liberates soluble TNF- α from the cell surface and thus releases a soluble cytokine.
B: cell-surface peptidases can generate biologically active peptides; e.g., angiotensin converting enzyme or ACE converts the decapeptide angiotensin I (ANG I) to the octapeptide ANG II, the principal active form.
C: cell-surface peptidases can degrade and inactivate neuropeptides; e.g., neutral endopeptidase (NEP) cleaves substance P (SP) at multiple sites to form inactive fragments.
D: soluble proteases can cleave protease-activated receptors (PARs) to expose a tethered ligand that binds and activates the cleaved receptor; e.g., the coagulation factor thrombin cleaves PAR$_1$ to activate the receptor.
E: soluble proteases can cleave PARs to remove the tethered ligand, generating a disabled receptor; e.g., cathepsin G from neutrophils cleaves PAR$_1$ to remove the tethered ligand and thereby prevent activation by thrombin.

Currently four PARs have been identified (PAR 1-4). Figure 5 shows the mechanism of cleavage (Figure 5A) and interaction of the tethered ligand with extracellular binding domain (Figure 5B). The functionally important domains in the amino terminus, second extracellular loop, and carboxy terminus are shown in part (Figure 5C). PAR-1 is expressed by platelets, fibroblast, endothelial cells and neurons, whereas PAR-2 is expressed by epithelial cells, neutrophils and neurons. PAR-1, -3, -4 are activated by

thrombin, and PAR-2 is activated by trypsin and a number of trypsin-like serine proteases. PAR-1 (and PAR-3) is activated by thrombin by a two-step process (Figure 6A). First thrombin binds to a hirudin-like domain; second, thrombin cleaves to expose the tethered ligand, which binds and activates the cleaved receptor. In some instances, protease binding to one receptor can facilitate cleavage of another receptor. PAR-3 is a cofactor for PAR-4 in murine platelets. Thrombin binds to the hirudin site of PAR-3, but PAR-3 does not signal in mouse platelets. The PAR-3 bound thrombin then cleaves and activates PAR_4 (Figure 6B).

A *Proteolytic cleavage*   **B** *Binding of tethered ligand*

C *Functionally important PAR domains*

Amino-terminus: Protease binding, cleavage and tethered ligand domains

PAR_1 PESKATNATLDPR SFLLRN PN DKYEPF WEDEEKNES
PAR_2 GTIQGTNRSSKGR SLIGKV DGTSHVTGKGVTVETCF
PAR_3 DTNNLAKPTLPIK TFRGAP PNS FEEFP FSALEEGWT
PAR_4 GDDSTPSILPAPR GYPGQV CANDSDTLELPDSSRAL

☐ tethered ligand
☐ hirudin-like site

Extracellular loop II: tethered ligand binding domains

PAR_1 QTIQVPGLNITTCHDVLNETLLEG
PAR_2 QTIFIPALNITTCHDVLPEQLLVG
PAR_3 QTIFIPALNITTCHDVLPEQLLVG
PAR_4 QTFRLARSDRVLCHDALPLDAQAS

Carboxy-terminus: desensitization, endocytosis

PAR_1 SSECQRYVYSILCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSIYKKLLT^{425}
PAR_2 SHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY^{397}
PAR_3 SKTRNHSTAYLTK^{374}
PAR_4 SAEFRDKVRAGLFQRSPGDTVASKASAEGGSRGMGTHSSLLQ^{385}

Figure 5   Structural and functional domains of PARs.

A *Protease binding to PARs*

Thrombin

▬ Tethered ligand
● Hirudin-like thrombin binding site

PAR_1
PAR_3

Signal transduction

B *Protease binding to PAR cofactors*

Thrombin

PAR_3 PAR_4

Signal transduction
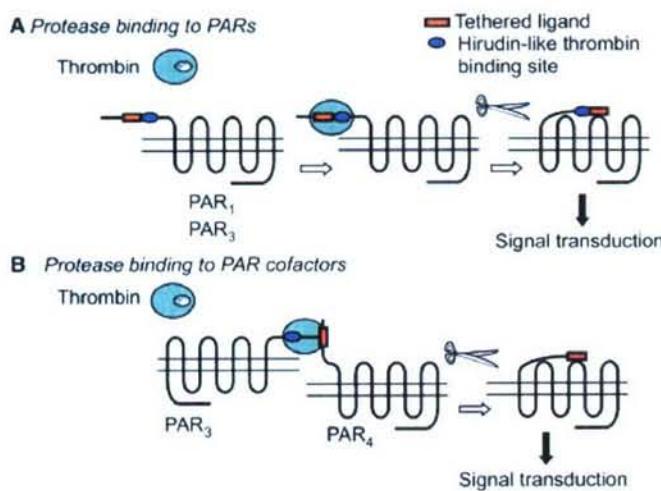
Figure 6   Mechanisms of activation and intermolecular cooperation of PARs.

PAR-2, expressed by numerous cells, is involved in inflammatory processes (leukocytes, endothelial cells, neurons). PAR-2 mediates acute inflammatory responses characterized by the development of edema and leukocyte infiltration.

PAR activation stimulates signal transduction mechanisms which may end in activation of transcription factors regulating the expression of tissue factor, adhesion protein, growth factors, cytokines, other ligand involved inflammation processes, and migration and proliferation of cells. PAR-1 agonist peptidases stimulate thrombin effects on cells but without receptor cleavage. The use of these agonist peptidases and PAR antagonsits might be useful for regulation of cells involved in blood coagulation, inflammation and tissue repair.

Bacterial proteases can also signal through PARs. *Porphyromonas gingivalis* is a major mediator of periodontitis in humans, and bacterial arginine-specific gingipains-R (RgpB) has been implicated in this disease. RgpB can activate PAR-1 and PAR-2 transfected cells and signals to an oral epithelial cell line to induce release of the powerful proinflammatory cytokine interleukin-6 [Lourbakos et al., 1998]. RgpB can also signal transfected cells expressing PAR1 and PAR4, and both proteases mobilize Ca^{2+} in platelets and induce aggregation [Lourbakos et al., 2001]. These results reveal a novel mechanism by which bacteria influence mammalian cells and could explain inflammatory reaction.

3.2.4   *Thrombin*

Thrombin, a trypsin-like serine protease, is a key enzyme of the blood coagulation system. It converts fibrinogen to fibrin and participates in the regulation of numerous physiological and pathophysiological processes such as blood coagulation and anticoagulation, thrombus formation and fibrinolysis, regulation of vascular tone, developmental processes, and also inflammation, tissue reparative processes, atherogenesis, carcinogenesis and Alzheimer disease. The polyfunctionality thrombin arises from its structure: besides the classical active site, it contains several additional sites called exosites or subsites of the additional recognition centers for substrate and receptors. Thrombin regulates inflammation, tissue repair and wound healing by cleaving and activating protease activated receptors (PARs; PAR-1 see Figure 4D) [Strukova, 2001]. The interaction of thrombin with PAR-1 of cultured vascular smooth muscle cells activated NF-κB and stimulated proliferation of these cells [Maruyama et al., 1997]. PAR activation by thrombin stimulates signal transduction mechanisms which may end in activation of transcription factors regulating the expression of tissue factors, adhesion proteins, growth factors, cytokines, other ligands involved in inflammation processes, and migration and proliferation of cells.

The binding of thrombin to thrombomodulin leads to the conversion of protein C to the serine protease activated protein C which impairs the inflammatory response.

It exerts an antithrombotic effect by inactivating factors Va and VIIIa, limiting the generation of thrombin (Figure 7). As a result of decreased thrombin levels, the inflammation response is reduced. Drotrecogin alfa (activated), or recombinant human activated protein C (rhAPC), has antithrombotic, anti-inflammatory and profibrinolytic properties [Bernard et al., 2001].

Treatment with activated protein C decreased inflammation, as indicated by decreases in interleukine-6 levels, a finding consistent with known anti-inflammatory activity of activated protein C. The anti-inflammatory activity of activated drotrecogin alfa may be mediated indirectly through the inhibition of the generation of thrombin, which leads to decreased activation of platelets, recruitment of neutrophils and degranulation of mast cells. Furthermore, preclinical study demonstrated that activated protein C has direct anti-inflammatory properties, including the inhibition of neutrophil activation

(reduction of rolling, see Figure 7) and production of cytokines by lipopolysaccharide-challenged monocytes, and E-selectin-mediated adhesion of cells to vascular endothelium.
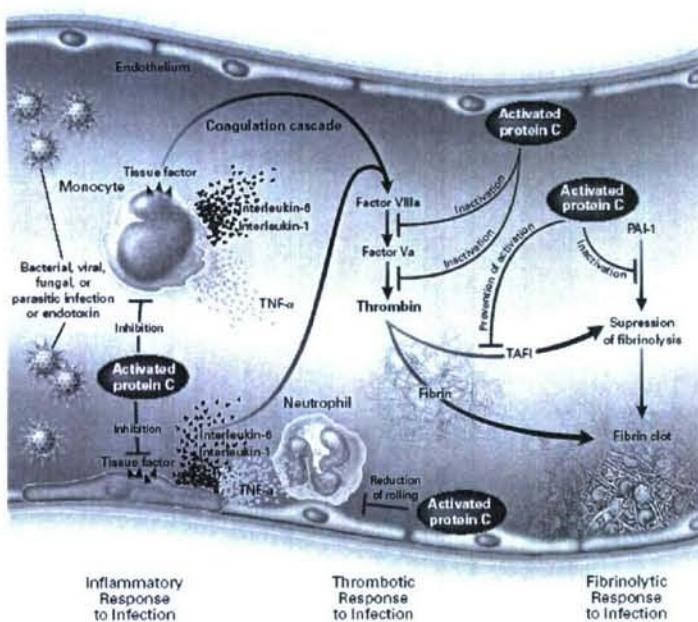

Figure 7   Actions of activated protein C in modulating the systematic inflammatory, procoagulant, and fibrinolytic host responses to infection [Bernard et al., 2001].

It was consistent with the antithrombotic activity of drotrecogin alfa activated that bleeding was the most common adverse event associated with the administration of the drug. Recent evidence indicates that the anticoagulant, activated protein C may be useful in the treatment of non-healing wounds by preventing excessive protease activity through inhibition of inflammation and selectively increasing MMP-2 activity to enhance angiogenesis and re-epithelialisation [Xue et al., 2006]. Drotrecogin alfa activated (recombinant human activated protein C) may be the first product to change the course of severe sepsis dramatically. This has not been proved beyond all reasonable doubt, but the evidence currently available demonstrates a considerable benefit when used for appropriate patients [Hughes, 2006]. Drotrecogin alfa (activated) (rhAPC) has been registered for use as adjuvant treatment in severe sepsis since 2001 under the trade name Xigris (®) essentially based on the results from one large clinical trial (the PROWESS trial). Administration of human recombinant activated protein C to patients with severe sepsis and high risk of death resulted in 19% relative risk reduction. In a recently published second randomized clinical trial (the ADDRESS trial), enrolling patients with severe sepsis but with less risk of death, no effect of treatment was shown, not even a trend to a positive effect in the subgroup of patients with a high risk of death that would match the present prescription label for Xigris (®). In addition, a large randomized, placebocontrolled trial with rhAPC in paediatric sepsis has recently been terminated prematurely because of lack of efficacy. The robustness of the data supporting the use of rhAPC in treating patients with severe sepsis may indeed be questioned. A confirmatory clinical trial is required before rhAPC can be used with confidence. The side-effects (increased risk of bleeding) and the cost of rhAPC are well documented but its efficacy is not [Gardlund, 2006].

3.2.5 Kallikrein/kinin system

The growth and proliferation of bacteria in human tissue/organs requires a continuous source of nutrients. Within the human host this is most easily obtained through proteolysis of either connective tissue proteins or plasma exudate. A more powerful mechanism involved the acquisition of plasma proteins. This occurs by deregulation of the kallikrein/kinin system and results in an overproduction of bradykinin (BK), a hormone that increases capillary permeability. In the normal cascade pathway, BK is formed through the action of plasma kallikrein, a serine protease (Figure 8).

During immunological reaction, charged surfaces, which may be derived from bacterial lipopolysaccharide, oligosaccharides, connective tissue proteoglycans or damaged basement membranes, enable factor XIIa. Once factor XIIa is present, pre-kallikrein can be cleaved into its active form, known as plasma kallikrein. This enzyme acts upon its preferred substrate, high molecular weight kininogen (HMWK), to release the nonapeptide bradykinin. Another kinin, Lys-bradykinin (kallidin, LBK) is produced via the action of an enzyme named tissue kallikrein on low molecular weight kininogen (LMWK). This enzyme is found in many tissues, either in the form of a precursor requiring activation or as an active enzyme. In contrast to plasma kallikrein which preferentially acts upon HMWK, tissue kallikrein can release kallidin from either HMWK or LMWK. Through the action of aminopeptidases, kallidin can subsequently be converted directly into bradykinin. This enzyme is present in both the plasma and on the surface of epithelial cells. Unlike HMWK which exists in the circulation as a complex with plasma prekallikrein, LMWK circulates freely [Chakravarty et al., 2005].

THE KININ-KALLIKREIN SYSTEM
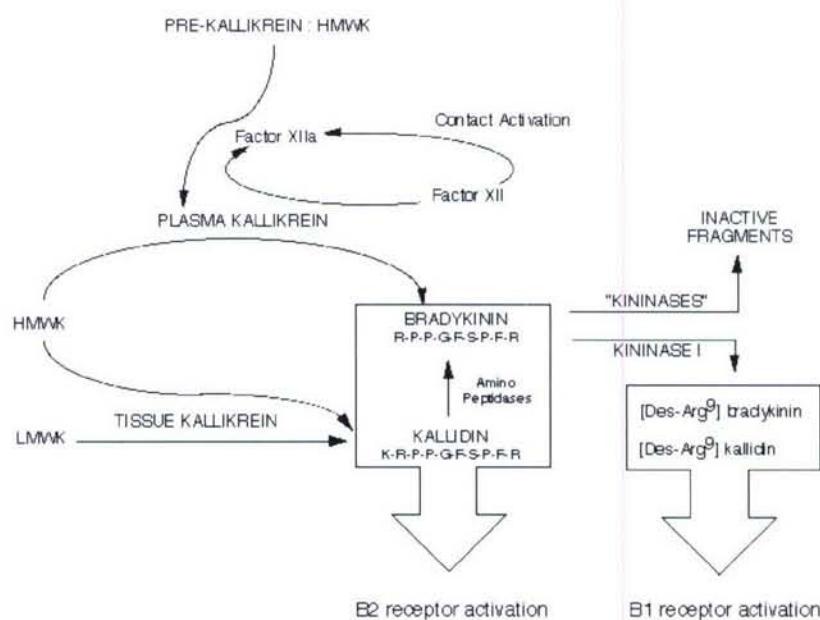
Figure 8   Diagram of the human kinin-kallikrein system including the native ligands for B_1-and B_2-receptor subtypes [Chakravarty et al., 2005]. B_2-receptor activation leads to pathophysiologies including pain, sepsis, asthma and inflammatory diseases. B_1-receptor activation leads to pathophysiology associated with prolonged inflammation.

In addition to tissue and plasma kallikrein, other serum and tissue proteases have a kinin forming capacity (Figure 9). Plasmin, which is responsible for lysis of the fibrin clot, releases not only bradykinin but also des-Arg^9-BK from HMWK, circulates in plasma as an inactive zymogen, plasminogen. The presence of plasminogen activators and their inhibitors is essential in controlling fibrinolysis [Moreau et al. 2005].

An imbalance between plasma kallikrein and its naturally occurring plasma inhibitors (endogenous C1 inhibitor, α_2-macroglobulin and antithrombin III) is associated with several disease states, such as hereditary angioedema (HAE), inflammatory bowel disease (IBD), systemic lupus, rheumatoid arthritis (RA), allergic rhinitis, and others. Both bradykinin and kallidin can be degraded by a variety of plasma and cell surface enzymes (kinases)[Ward, 1991]. The most widely recognized of these enzymes are kinase I, kinase II (angiotensin converting enzyme, ACE) and carboxypeptidase N. In plasma, kinase I cleaves the C-terminal arginine from both bradykinin and kallidin to form [des-Arg^9] kinins. These [des-Arg^9] kinins are known to act as agonists of B_1-receptors which are present in some species and have been implicated in the pathophysiology associated with prolonged inflammation [Perkins et al., 1992]. The initial digestion of kinins by kininase II results in the removal of the terminal phenylalanine-arginine, leaving R^9-F^8-BK or des- R^9-F^8-LBK. Subsequent cleavage result in additional kinin fragments, including the pentapeptide RPPGF [Majima et al., 1996].
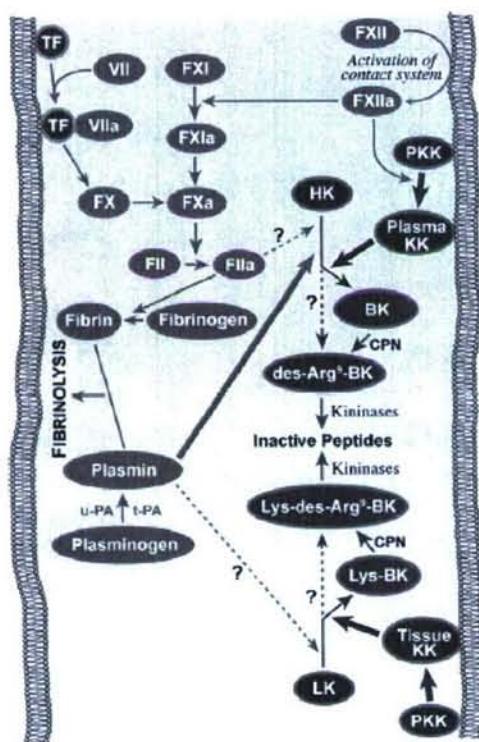
Figure 9   The kinin-forming systems. The kallikrein-kinin system and its interactions with both intrinsic and extrinsic coagulation cascades and fibrinolysis. Solid lines are established pathways, whereas dashed lines are speculative or experimental activation pathways. TS: tissue factor; PKK: prekallikrein; HK: high-molecular-weight kininogen; LK: low-molecular-weight kininogen; BK: bradykinin; CPN: carboxypeptidase; t-PA: tissue plasminogen activator; u-PA: urokinase plasminogen activator [Moreau et al. 2005].

3.2.5.1   *B₁-receptor and B₂-receptor*

Nearly all cells express kinin receptors (B_2-receptors) which mediate the activities of both bradykinin and kallidin. The B_2-receptor is normally constitutively expressed. The stimulation of B_2-receptor (and B_1-receptor) leads to the activation of phospholipase Cβ and to the production of inositol 1,4,5-triphosphate, with consequent increase of intracellular calcium concentration [Campos et al., 2006]. This cascade of reactions leads to relaxation of venular smooth muscle and hypotension, increased vascular permeability, contraction of smooth muscle of the gut and airway leading to increased airway resistance, stimulation of sensory neurons, alteration of ion secretion of epithelial cells, production of nitric oxide, release of cytokines from leukocytes and eicosanoids from various cells. Because this spectrum of activity, kinins have been implicated in many pathophysiologies including pain, sepsis, asthma, symptoms associated with rhinoviral infection, rheumatoid arthritis, and a wide variety of other inflammatory diseases. At cellular level, BK stimulation of the B_2-receptor leads to rapid desensitization of the receptor response as determined both by PI hydrolysis and the increase in intracellular Ca^{2+}. The mechanism of B_2-receptor desensitization involves phosphorylation of specific serines and threonines in the C-terminal tail [Leeb-Lundberg et al., 2005].

The kinin B_2-receptor ligand bradyzide is about 1000 times more potent to the rat B_2-receptor than on the human B_2-receptor, its affinity for the rabbit ortholog being intermediate. So, quite large species differences in affinity were noted within this class of drugs. A series of analogues in which the diphenylmethyl moiety of bradizyde has been substituted with dibenzosuberane, have been reported to gain potency at the human B_2-receptor, with some loss of affinity for the rat receptor [Marceau et al., 2003].

Given the protective B_2-receptor mediated actions in the cardiovascular and renal systems and because a large body of animal data suggests that such drugs may precipitate cardiovascular accidents in predisposed individual, the development of B_2-receptor antagonists as anti-inflammatory and analgesic drugs may be a potential concern, even though clinical evidence for this notion is lacking [Leeb-Lundberg et al., 2005; Heitsch, 2003].

Like the B_2-receptor, the B_1-receptor is heptahelical receptor, but unlike B_2-receptors, it is not widely expressed in normal tissue but is highly inducible by inflammatory mediators like bacterial lipopolysaccharide (LPS) and cytokines and does not desensitize after agonist binding [Pesquero et al., 2000]. Because of their inducible pattern, B_1-receptors do not seem to be housekeeping molecules and it might be possible that therapeutic use of selective kinin B_1-receptor antagonists would not produce undesirable side effects [Campos et al., 2006].

The constitutive expression of B_2-receptors on vascular endothelial cells underlies the initial inflammatory response leading to edema, plasma extravasation and the ensuing generalized inflammatory response, which includes local tissue generation of high levels of kinins. The generalized inflammation including enhanced release of local inflammatory cytokine mediators (TNF-α, IL-1) induces the local expression of B_1-receptors on various cell types, thereby propagating the inflammatory response, including the associated activation of nociceptors. Thus this cascade of events has led to the view that B_2-receptors are involved in the initial inflammatory response followed by the appropriate induction of B_1-receptors to potentiate and mediate longer term inflammation and pain. Contributing to this sequence is the kinetics of receptor downregulation whereby B_2-receptors appear to be readily phosphorylated and internalized, whereas B_1-receptors have a longer functional half-life on the surface of the cell [Leeb-Lundberg et al., 2005].

3.2.5.2   *Inhibitors of kallikrein/kinin system*

Small molecule inhibitor, analog 11, demonstrated the best potency and selectivity
profile for plasma kallikrein versus related serine proteases [Young et al., 2006]. It was
tested in rats and could be further developed for treatment of inflammatory disorder.
However, the total production of bradykinin is the sum of the activity of plasma
kallikrein and plasmin. When only plasma kallikrein is inhibited, the activity of plasmin
still results in production of bradykinin. Therefore, to treat the effects of bradykinin,
bradykinine receptor antagonists are a better option. More inhibitors for plasma
kallikrein are discussed in Paragraph 5.5.

The use of mice or rats as animal models for studying the kallikrein-kinin system can
give problems, because difference in specificity between rodent enzymes and enzymes
of other mammals exists. Inhibitors that were found to inhibit human tissue kallikrein in
the nM range are weak inhibitors of mice and rat tissue kallikrein [Fogaca et al., 2004].
A bradykinin antagonist (NPC 18884) showed divergent potency when assayed in
different species. In particular in a model of bradykinin-induced hypotension in rats and
rabbits, it appears to have no activity. Likewise, it did not block bradykinin-induced
contraction of the isolated guinea pig ileum. However, it binds to the human B_2-receptor
[Chakravarty et al., 2005].

HOE 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK, Icatibant) is a representative of the
second generation of peptide B_2-receptor antagonists [Hock et al., 1991] and has been
exploited in more than 700 research papers and several clinical studies. HOE 140 is
potent and competitive at the human B_2-receptor, but insurmountable and atypically
induces a slow B_2-receptors endocytosis of the rabbit B_2-receptor. These examples
showed that there are both practical and theoretical consequences for such large species
selectivity: the preclinical development of drugs ultimately designed for human use
becomes more difficult if the pharmacodynamic actions of the drug candidate cannot be
studied in animal models. Mutagenesis and chimera constructions to 'humanize' parts
of B_2-receptor sequence from laboratory animals are possible avenues for future
investigations and could provide clues on the similarity or differences in the docking
sites of various chemical classes of non-peptide antagonists to B_2-receptors
[Marceau et al., 2003]. In Appendices K and L, the pharmacological and clinical
application of, respectively, kinin B_1- and B_2-receptor ligands are shown.

Compound 11 was synthesized as a B_1-receptor antagonist [Morissette et al., 2004].
It showed to be more potent in rat than in rodent. Results support that compound 11 is a
potent and highly selective antagonist suitable for further investigations of the role of
the kinin B_1-receptor in models of inflammation, pain, and sepsis based on the rabbit.

3.2.5.3   *Infectious diseases and the kallikrein-kinin system*

Many bacterial pathogens elicit proteolytic activities that mimic the proteolytic
activities in the kallikrein/kinin cascade either directly or indirectly releasing BK from
kininogens [Maeda and Yamamoto, 1996]. At infected sites, this would result in the
development of edema. It is therefore likely that much edema found at inflammatory
sites during infection by these pathogens is also a result of BK production. BK
generation at the infection focus may facilitate the intravascular dissemination of
pathogens [Maruo et al., 1998]. It was also shown by Sakata et al. (1996) that bradykin
generation triggered by *Pseudomonas* proteases, facilitates invasion of the systemic
circulation by *Pseudomonas aeruginosa*.

Injection of LPS from *E.coli* into dorsal skin of rats caused a dose-dependent increase in
vascular permeability and that this increase caused by LPS was attenuated by pre-
treatment with the B_2-receptor antagonist HOE 140. Another therapeutic approach of
sepsis could be the use of the pentapeptide RPPGF [Majima et al., 1996].

This metabolic fragment of bradykinin prevents the deleterious effects of endotoxin (LPS) in both anesthetized rats and in isolated rat aortic segments [Morinella et al., 2001].

LPS is a classical signal for B_1-receptor upregulation. Selective B_1-receptor antagonists can reduce inflammatory and haemodynamic events following exposure to LPS, and gene deletion of B_1-receptor prevents endotoxic shock in mice. Mice overexpressing B_1-receptors are more susceptible to endotoxic shock. Recent evidence suggests that B_1-receptors are induced by *Staphylococcus aureus* and *Burkholderia cenocepacia* infection [Campos et al., 2006].

In a study done by Monteiro et al. (2006) an infection model (mouse model of subcutaneous infection by *Trypanosoma cruzi*) was described where type 1 immunity (Th1 response) was vigorously up-regulated by bradykinin, an innate signal whose levels in peripheral tissues were controlled by an intricate interplay of TLR2, B_2-receptor and angiotensin converting enzyme. Th1-type cytokines (IL-12, IFN-γ) tend to produce the proinflammatory responses responsible for killing intracellular microorganisms. The effect of IL-12 administration to infected mice was already shown in 1996 by Kawakami et al. Systematic administration of IL-12 to mice with pneumonia due to *C. neoformans* resulted in improved pulmonary clearance, decreased dissemination to the brain and increased survival. In excess, Th2 responses will counteract the Th1 mediated microbicidal action. Excessive proinflammatory responses can lead to uncontrolled tissue damage so an optimal scenario would therefore seem to be that humans should produce a well balanced Th1 and Th2 response, suited to the immune challenge [Berger, 2000].

3.2.6   *Caspases*

Caspases are a group of cysteine proteases, enzymes with a crucial cysteine residue that can cleave other proteins after an aspartic acid residue, a specificity which is unusual among proteases. The name 'caspase' derives from this characteristic molecular function: *c*ysteine-*asp*artic-*a*cid-proteases. Human caspases functionally segregate into two distinct subfamilies: those that involved in cytokine maturation (caspase-1, -4, -5 and -11) and those involved in cellular apoptosis (caspase-2, -3, -6, -7, -8, -9, -10) [Saleh et al., 2004]. Caspases are synthesized as zymogens with a prodomain of variable length followed by a large and a small subunit. The large prodomains contain a class of related protein recruitment motifs, such as the caspase recruitment domain (CARD, caspase-1, -2, -4, -5, -9, -11, and -12) and the death effector domain (caspase-8 and -10). These prodomains allow recruitment in large protein complexes, eventually leading to caspase auto-activation and initiation of the apoptotic and inflammatory patways (Figure 10).
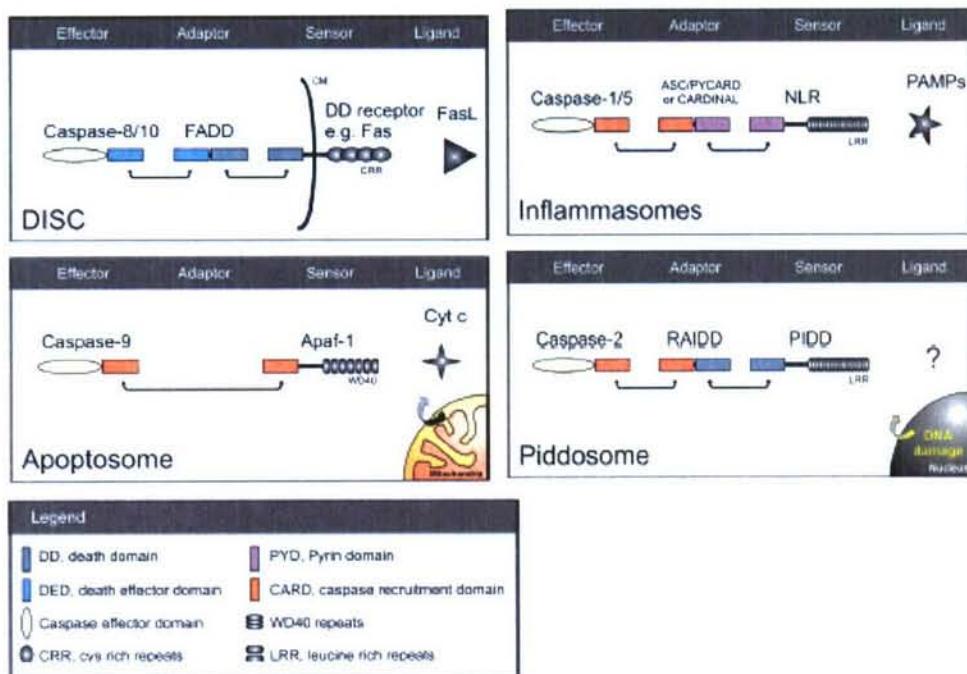
Figure 10   Overview of the main protein complexes leading to the activation of large prodomain caspases.
             Complex formation is initiated by different ligands and sustained by several interaction motifs
             harbored in complex-residing proteins. The ligand-sensing motifs (leucine-rich repeats, WD40
             repeats, and CRRs) initiate the formation of oligomers. Death domain and death effector
             domain or CARD-CARD homotypic interactions are crucial for the recruitment and activation
             of either caspase-8 in the DISC or caspase-9 in the apoptosome, respectively. Caspase-1 or -5 is
             activated in the different inflammasomes using different adaptors such as ASC/PYCARD or
             CARDINAL, depending on the type of onflammasome. Several compounds that lead to
             inflammasome activations, called PAMPs, were identified (RNA, LPS, peptidoglycans).
             Caspase-2 is activated in the PIDDosome, using the adaptor molecule RAIDD upon DNA
             damage. How the nuclear damage triggers PIDDosome formation is currently not clear
             [Lamkanfi et al. 2006].

The death-inducing signaling complex (DISC) containing caspase-8 is formed at the
intracellular domain of death receptors, such as Fas, TRIAL receptor and TNF
receptor 1. Fas-associated death domain containing protein (FADD) plays a crucial role
in the recruitment and activation of caspase-8 and -10 in the DISC. Mitochondrial
damage results in the release of cytochrome c, triggering the assembly of the
apoptosome complex that directly recruits caspase-9 [Lamkanfi et al., 2006].
In mammals a large family of BH3-only proteins is distributed throughout the cell to
sense apoptotic stress signals. Upon receiving apoptotic stimuli, the BH3-only proteins
transduce the signal to mitochondria. Through complex actions involving Bak and Bax,
cytochrome c is released from the intermembrane space of mitochondria into the
cytoplasm, where it binds to and activates Apaf-1. Then, the binary complex of Apaf-1
and cytochrome c binds its critical cofactor dATP or ATP, forming a multimeric
complex dubbed the apoptosome. The only known function of the apoptosome is to
recruit and to facilitate activation of caspase-9. Once activated caspase-9 stays
associated with the apoptosome as a holo-enzyme to maintain its catalytic activity, as
caspase-9 in isolation is marginally active. The primary target of the caspase-9 holo-
enzyme is caspase-3, one of the most deleterious effector caspases (Figure 11)
[Shi, 2004].

In vitro, caspase-2 is part of a high molecular weight complex containing the p53-induced death domain protein (PIDD). Because the p53 tumor suppressor can elicit apoptosis in response to DNA damage, it was suggested that this PIDDosome complex is formed under DNA-damaging conditions and functions as a platform for caspase-2 activation. The inflammatory caspases-1 and -5 have also been shown to be recruited to a number of protein platforms, named inflammasomes. Typically, the platform of these complexes consists of members of the NACHT-LRRs (NLRs). NLRs are intracellular pathogen-recognition receptors that initiate inflammatory signaling and/or cell death. These intracellular receptors are activated by different pathogen-associated molecular patterns or PAMPs [Lamkanfi et al., 2006].
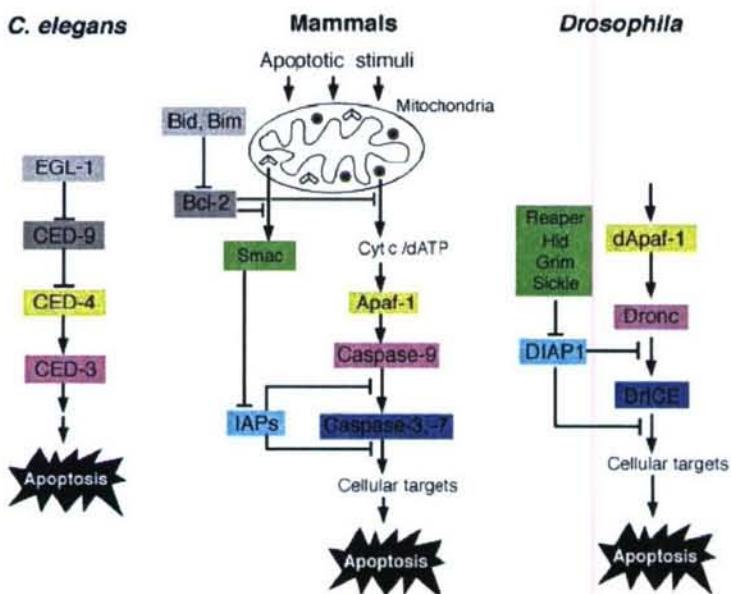
Figure 11   A conserved apoptotic pathway in *C. elegans*, mammals, and *Drosophila*. Caspase-9 in mammals and Dronc in Drosophila are initiator caspases, whereas caspases-3 and -7 in mammals and DrICE in *Drosophila* belong to effector caspases. CED-3 in *C. elegans* is both the initiator and the effector caspase [Shi, 2004].

Caspase-12 is phylogentically related to cytokine maturation caspases, but it has been proposed as mediator of apoptosis. Failure of apoptosis is one of the main contributions to tumour development and autoimmune diseases; this coupled with the unwanted apoptosis that occurs with ischaemia or Alzheimer's disease, has boomed the interest in caspases as potential therapeutic targets since they were discovered in the mid 1990s. Caspases function in both apoptosis and inflammatory cytokine processing and thereby have a role in resistance to sepsis. A novel role for a caspase was described in dampening responses to bacterial infection [Saleh et al., 2006]. In mice, gene-targeted deletion of caspase-12 renders animals resistant to peritonitis and septic shock. The resulting survival advantage was conferred by the ability of the caspase-12-deficient mice to clear bacterial infection more efficiently than wild-type littermates. Caspase-12 dampened the production of the pro-inflammatory cytokines interleukin (IL)-1beta, IL-18 (interferon (IFN)-gamma inducing factor) and IFN-gamma, but not tumour-necrosis factor-alpha and IL-6, in response to various bacterial components that stimulate Toll-like receptor and NOD pathways. The IFN-gamma pathway was crucial in mediating survival of septic caspase-12-deficient mice, because administration of neutralizing antibodies to IFN-gamma receptors ablated the survival advantage that otherwise occurred in these animals. In mice, caspase-12 deficiency confers resistance to sepsis

and its presence exerts a dominant-negative suppressive effect on caspase-1, resulting in enhanced vulnerability to bacterial infection and septic mortality. Caspase-12 is found in around 20% of people of African descent, and was entirely lost from all other ethnicities around 60,000 years ago. Caspase-12 is therefore only a target enzyme to develop treatment that may help strengthen the immune system of those people unfortunate enough to have the caspase-12 gen product [Kachapati et al., 2006].
The inhibitor of apoptosis (IAP) family of proteins suppresses apoptosis by interacting with inhibiting the enzymatic activity of both initiator and effector caspases. Several distinct mammalian IAPs including XIAP, c-IAP1, c-IAP2, and ML-IAP, have been identified, and they all exhibit antiapoptotic activity in cell culture. The functional unit in each IAP protein is the baculoviral IAP repeat (BIR), which contains approximately 80 amino acids folded around a zinc atom. Most mammalian IAPs have more than one BIR domain, with the different BIR domains performing distinct functions.
For example, in XIAP, the third BIR domain (BIR3) potently inhibits the catalytic activity of caspase-9, whereas the linker sequences immediately preceding the second BIR domain (BIR2) selectively targets caspase-3 or -7.

3.3 Bacterial proteases involved in inflammation processes

Bacteria have different ways to affect the health of a human being:
- damage membranes;
- inhibit protein synthesis;
- activate second messenger pathways;
- activate immune respons;
- proteases.

In Appendix D, an overview of the organism/toxin involved in these processes is described. Because the aim of this report is to select peptidases that could act as a target for generic peptidase inhibitors in therapy to infectious agents, the bacterial proteases will be discussed in more detail. Metallo-, cysteine- and serineproteases are widely spread in many pathogenic bacteria and play a critical role related to colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection. Table 3 illustrates a list of proteolytic activities that have been detected in principal and opportunistic pathogens.
A detailed list of bacterial serine/cysteine and metalloproteases can be found in respectively, Appendices E and F.

Bacterial proteases can have different approaches to interact with human targets. Potential host targets for bacterial proteinases are [Travis and Potempâ, 2000]:
- *Inactivation of host proteinase inhibitors*
  Approximately 10% of the proteins in human plasma are utilized to regulate proteolytic events in tissues, with most of them belonging to the serpins superfamily of proteinase inhibitors. During the past several years, bacterial proteinases have been found that specifically inactivate human plasma serpins.

- *Degradation of connective tissue components*
  Degradation of elastin and collagen during tissue destruction is primarily due to the action of host-derived proteases released by macrophages and neutrophils.

However indirect mechanisms might be involved in tissue destruction:

a activation of host MMP as is shown for enzymes of *P. aeruginosa* and *V. cholerae*

b plasmin(ogen)-binding and activation which has been noted for organisms including *Borrelia burgdorferi*, *Streptococci* sp. and *Staphylococci* sp.

c inactivation of plasma proteinase inhibitors, which allow host proteases to act in an unregulated manner.

d transient activation of chemotactic pathways to recruit phagocytic cells to inflammatory sites.

Table 3   Extracellular proteolytic activities produced by principal and opportunistic bacterial pathogens.

Organism	Catalytic type and proteinase family affiliation
B. fragilis	M10
Clostridium spp.	M4, M9, C11
L. pneumophila	M4
P. gingivalis	M13, M24, C1, C10, C25, S9
P. mirabilis	M10
P. aeruginosa	M4, M10, M23
S. marcescens	M10, S8
Staphylococcus spp.	C1, M4,
S. pyogenes	C10, S8
T. denticola	S8
Vibrio spp.	M4
B. anthracis	M34
Clostridium tetani	M27
Clostridium botulinum	M27

M4: thermolysin; M9: microbial collagenase; M10: serralysin; M13: neprilysin; M23: beta-lytic metallopeptidase; M24: methionyl aminopeptidase 1; M27: tentoxilysin; M34: anthrax lethal factor; C1 papain; C10: streptopain; C11: clostripain; C21: tymovirus peptidase; S8: subtilisin Carlsberg; S9: prolyl oligopeptidase.

There are bacterial pathogens that do secrete proteinases whose function is to directly degrade host tissue components (Table 4).

Table 4   Bacterial pathogens that secrete proteases which degrade host tissue components.

Organism	Protease
Clostridium perfringens, Clostridium histolyticum	clostripain, clostridial collagenase, lambda toxin
P. aeruginosa	pseudolysin, aeruginolysin
Serratia marcescens	serralysin
B. fragilus	fragilisin
Staphylococcus epidermidis	metalloelastase
Vibrio vulnificus	vibriolysin

- *Deregulation of immune system*

  Proteinases from different classes with only IgA1-degrading activities have been specifically isolated from pathogens such as *Haemophilus influenza*, *P. aerginosa*, *Neisseria gonnorrhoeae*, *Neisseria meningiidis*, *Proteus mirabilus*, *Streptococcus sanguis* and *Streptococcus pneumoniae*.

  Gingipains are trypsin-like cysteine proteinases produced by *Porphyromonas gingivalis*, a major causative bacterium of adult periodontitis. Rgps (HRgpA and RgpB) and Kgp are specific for -Arg-Xaa- and -Lys-Xaa- peptide bonds, respectively. Rgps enhanced vascular permeability through prekallikrein

activation or direct bradykinin release in combination with Kgp. This Rgp action is potentially associated with gingival edema and crevicular fluid production. Rgps activate the blood coagulation system, leading to progression of inflammation and consequent alveolar bone loss in the periodontitis site. Rgps also activate protease-activated receptors and induce platelet aggregation, which, together with the coagulation-inducing activity, may explain an emerging link between periodontitis and cardiovascular disease. Kgp is the most potent fibrinogen/fibrin degrading enzyme of the three gingipains in human plasma, being involved in the bleeding tendency at the diseased gingiva. Gingipains stimulate expression of matrix metalloproteinases (MMPs) in fibroblasts and activate secreted latent MMPs that can destroy periodontal tissues. Gingipains degrade cytokines, components of the complement system and several receptors, including macrophage CD14, T cell CD4 and CD8, thus perturbing the host-defense systems and thereby facilitating sustained colonization of *P. gingivalis* [Imamura et al., 2003].

- *Deregulation of proteinase cascade*
  A common feature of all cacade systems in mammalian species (complement, kalikrein/kinin, coagulation and fibrinolytic pathways) is the strict requirement for zymogen activation by cleavage after specific Arg-X residues by serine proteases. This together with presence of controlling inhibitors of the same enzymes ensures that each system is tightly regulated. Pathogens provide their own site of enzymes (most often cysteine proteinases) that can inactivate inhibitors.
- *Interruption of host cell communication*
- *Other functions of pathogen-derived proteinases*
  Bacterial pathogens secrete a number of proteolytic enzymes that only attack highly specific substrates within human tissues and cause many symptoms associated with specific infections. The most well known examples are those that are due to bacterial toxins and which function by degrading and inactivating very specific protein substrates, neurotoxins of *C. botulinum* and *C. tetani*, and the lethal factor of *B. anthracis*.

3.4 Bacterial metalloproteases

Bacterial metalloproteases fall into three families; thermolysin, serralysin and neurotoxin families (Figure 4). Thermolysin and several related bacterial metalloproteases, including bacillus sp. neutral proteases, *Pseudomonas aeruginosa* elastase, *Vibrio cholerae* and *Vibrio vulnificus* protease and *Legionella pneumophila* protease, have two well-conserved regions involved in zinc binding. The short zinc binding motif is contained within the longer consensus HEXXHXB, while the glutamic acid third ligand lies 25 residues towards the C-terminal from the zincin motif in the consensus sequence GXBNEXBSD [Miyoshi and Shinoda, 2000]. The metzincins have longer zinc binding consensus sequence HEBXHXBGBXH which contain three of the zinc ligands. In addition this superfamily has a methionine-containing turn of similar conformation (the Met-turn).

The neurotoxins produced by *Clostridium botulinum* and *C. tetani* are known to inhibit the release of the neurotransmitter, acetylcholine. The third zinc ligand is most likely a conserved glutamic acid, which is 40 residues towards the C-terminal from the HEXXH motif.

The *Bacillus anthracis* lethal factor is a Zn^{2+}-endopeptidase specific for the MAPK-kinase family of proteins. In addition to the HEXXH motif, a glutamic acid (Glu 735) function as a third zinc ligand. Such zinc coordination resembles that of the thermolysin family of metalloproteases, but analysis of the second shell of residues surrounding the zinc atom reveals the presence of a tyrosine residue (Tyr-728) absent in thermolysin. A similar tyrosine is instead present in the clostridial metalloprotease neurotoxins [Tonello et al., 2004].

In Table 3 and 4, an overview is given of bacterial proteases which are produced by principal and opportunistic bacterial pathogens. Characteristics of these proteases can be found in literature. However, instead of doing a literature search to find information about potential generic inhibitors, another tool can be used. This tool, the MEROPS database will be discussed in the next chapter.

4   MEROPS

4.1   The MEROPS database and A-list/B-list NIAID Priority Pathogens

To find generic bacterial peptidases that could possibly serve as target for medical countermeasures in a biological attack, the MEROPS database was used [Rawlings et al., 2006; MEROPS: the peptidase database; http://merops.sanger.ac.uk/). MEROPS is an information resource for peptidases and the proteins/molecules that inhibit them. The MEROPS database uses a hierarchical, structure-based classification which is based on that of Rawlings and Barrett (1993), in which the catalytic type of the protein represents the top level in the hierarchical classification. According to this rule, the peptidases can be divided into clans based on three-dimensional protein folding and into families based on evolutionary relationships of the primary sequence. Today, a lot of bacterial, archaeal, protozoal, fungal, plant, animal and viral genome sequences, coding for proteases, are known.

For the A-list and B-list priority pathogens, which are listed on the NIAID website, a search was done in the MEROPS database. In Table 5, pathogens are displayed, from which the total genome sequence is unraveled. When the total genome sequence is present, all sequences with homology to peptidase sequences can be found.

Table 5   Genomes of Priority Pathogens present in the MEROPS database version 7.3.

A-list Priority Pathogens	B-list Priority Pathogens
B. anthracis	B. melitensis
Y. pestis	V. cholerae
F. tularensis	B. abortus
	B. suis
	R. prowazekii
	B. mallei
	B. pseudomallei
	C. jejuni
	C. burnetii
	V. parahaemolyticus
	V. vulnificus
	L. monocytogenes
	E. coli O157
	S. typhi
	S. flexneri

By scoring the presence of generic peptidases in the genomic sequences of all NIAID Priority Pathogens of Table 5, nine peptidases from distinct Clans/Families were found. These peptidases are shown in Table 6. However, the use of generic inhibitors of these peptidases during medical counter measures could possibly lead to the inhibition of human proteases with the same homology. Therefore, peptidases which can also be found in the human genome are ruled out (see Table 6) as a putative target in medical counter measures. A more detail description of the peptidases from Family A8 (Clan AC) can be found in Paragraph 4.2.1.

Table 6   Bacterial peptidases present in NIAID Priority Pathogens (shown in Table 5).

Clan	Family	Type of peptidase	Present in human genome
AC	A8	Signal peptidase II	no
MG	M24	Methionyl aminopeptidase 1	yes
MH	M20	Glutamate carboxypeptidase	yes
MK	M22	O-sialoglycoprotein peptidase	yes
MM	M50	S2P peptidase	yes
PB	T1	Archaean proteasome	yes
SF	S26	Signal peptidase I	yes
SJ	S16	Lon-A peptidase	yes
SK	S14	Peptidase Clp	yes

If a sequence homologue of a peptidase involved in tissue degradation is present in
human and bacteria, it would be an ideal target for inhibitor synthesis.
In Appendix J the biological activity of the peptidases shown in Table 6 is described.
None of these 'generic' peptidases found in human and bacteria are involved in tissue
degradation so the option to select a generic human/bacterial protease inhibitor that
stops tissue degradation can be ruled out.

4.2   Bacterial peptidases present in all A-list NIAID Priority Pathogens

In a more detailed examination of peptidases in the MEROPS database, absent in the
human genome but present in only A-list NIAID Priority Pathogens (*B. anthracis*,
Y. pestis, and *F. tularensis*), is shown in Table 7. In this case, a total of 6 peptidase
types were found. These peptidases were also screened in the B-list NIAID Priority
Pathogens. In Appendix G a list is shown which shows the presence or absence of
selected proteases in the B-listed group.

Table 7   Peptidase count of bacterial peptidases that are not present in the human genome.
          In Appendix G, an overview of the count of B-list pathogens is given.

Clan	Family	Peptidase count		
		Count Total N(total) = 18	Count A-list N(total) = 3	Count B-list N(total) = 15
AC	A8	18	3	15
SE	S11	17	3	14
SF	S24	17	3	14
U-	U32	16	3	13
AD	A24A	14	3	11
MD	M15	14	3	11

A description of the peptidases from these Families/Clans is described below. If an
inhibitor of this protease is known, it will be mentioned. A more detailed description
can be found in the MEROPS database. There, for each protease a summary page
describes the classification and nomenclature, and provides links to supplementary
pages showing sequence identifiers, the structure (if known), inhibitors (if known)
literature references and more.

4.2.1   *Hum Peptidase family A8; an endopeptidase, the bacterial signal peptidase II*
The biological function of the signal peptidase II is to remove the signal peptide from the N-terminus of the murein prolipoprotein, an essential step in the production of the bacterial cell wall. Homologues are known from nearly every bacterial genome so far completely sequenced. A few bacteria, including *Pseudomonas fluorescens* and *Staphylococcus epidermidis*, contain two family A8 homologues.

It is known that the cyclic pentapeptide antibiotic globomycin is a potent noncompetitive inhibitor. Pepstatin, Tos-Arg-OMe and mercuric chloride also inhibit at concentrations below 1 mM [Sankaran, 2004].

4.2.2   *Peptidase family S11; a serine-type D-Ala-D-Ala carboxypeptidases*
The biological function of the peptidases of family S11 are mainly involved in the synthesis of bacterial cell walls, cleaving the D-Ala-D-Ala crosslinks in the cell wall peptidoglycans. Many of the enzymes are also penicillin-binding proteins (PBPs).

The molecular structure of family S11 is included in clan SE as the protein fold of the peptidase unit for members of this family resembles that of the type example of family S12, D-Ala-D-Ala-carboxypeptidase B (S12.001). The peptidases of family S11 contain two domains - one of alpha helices and one containing an alpha-beta sandwich.
The active site residues reside on a helix that crosses the cleft between the two lobes. The all-alpha helix domain, containing the third catalytic residue in the motif Ser-Xaa-Asn, forms one side of the cavity whereas the other side is formed by the third strand of the beta sheet containing a Lys-Thr-Gly motif.

Antibiotics of the beta-lactam family inactivate some members of family S11 through acylation of the active site serine.

4.2.3   *Peptidase family S24; a two-domain proteins.*
The biological function of the repressor LexA is in the SOS response leading to the repair of single-stranded DNA within the bacterial cell. The SOS system represents a global response to DNA damage that upregulates genes involved in DNA repair and cell survival. The SOS response is governed by the LexA and RecA proteins. The LexA protein binds to operator sites of SOS-regulated genes, effectively repressing their expression. Conversely, the presence of DNA lesions activates RecA, which promotes the autocatalytic cleavage of LexA at a specific Ala-Gly bond. Cleaved LexA is unable to bind DNA, leading to the derepression of SOS genes.
UmuD is also involved in the SOS response, but here cleavage releases and activates the DNA polymerase UmuC which forms a complex with uncleaved UmuD [Reuven et al., 1999]. Cleavage of the bacteriophage lambda repressor CI leads to prophage induction: the prophage is integrated into the host genome, but expression is blocked by repressor CI until the SOS response leads to CI autolysis, derepression of the prophage and expression of the lytic genes and growth of the virus [Little, 2004].

The molecular structure of the peptidase family S24 contains two-domain proteins that undergo autolysis, separating the functional domains.

There are no molecules known to inhibit this type of peptidases.

4.2.4   *Peptidase family U32; endopeptidases from bacteria*
The biological function of the PrtC peptidase (U32.001) was reported to degrade soluble and reconstituted fibrillar type I collagen, heat-denatured type I collagen, and azocoll, but not gelatin or a synthetic collagenase substrate [Kato et al., 1992]. Activity of the homologue from *Helicobacter pylori* has been reported, but not characterized in detail [Kavermann et al., 2003].

The molecular structure of the PrtC peptidase ran as a band of 35 kDa in SDS gel electrophoresis, and the native enzyme behaved as a dimer in gel filtration chromatography [Kato et al., 1992]. The deduced amino acid sequence shows no relationship to those of peptidases in any other family.

The PrtC peptidase was inhibited by EDTA and thiol-blocking agents [Kato et al., 1992].

4.2.5   *Peptidase family A24; a membrane-inserted endopeptidases*
The biological function of type 4 prepilins are required for functions including type IV pilus formation, toxin and other enzyme secretion, gene transfer and biofilm formation [LaPointe & Taylor, 2000]. The pilins can perform these functions only after processing by a prepilin peptidase.

The molecular structure of the peptidases of subfamily A24A contains eight predicted transmembrane domains (five of which are included in the peptidase unit).

There are no molecules known to inhibit this type of peptidases.

4.2.6   *Peptidase family M15; metallopeptidases*
The biological function of the peptidases of family M15 is involved in bacterial cell wall biosynthesis and metabolism. They include zinc-dependent D-Ala-D-Ala carboxypeptidases and dipeptidases, and bacteriophage endolysins. The structure of the peptidoglycan polymer that constitutes the cell wall is stabilized by a cross-linking peptide that contains D-amino acids. The cross-linking peptide is synthesized as a precursor with an additional C-terminal D-Ala residue. The removal of the C-terminal D-Ala by the carboxypeptidase prepares the precursor for incorporation into the cell wall. Vancomycin-resistant *enterococci* are pathogenic bacteria that attenuate antibiotic sensitivity by producing peptidoglycan precursors that terminate in D-Ala-D-lactate rather than D-Ala-D-Ala. A key enzyme in antibiotic resistance is the metallodipeptidase VanX that reduces the cellular pool of the D-Ala-D-Ala dipeptide so that only the resistant D-Ala-D-lactate is incorporated into the cell wall [Lessard & Walsh, 1999].

The molecular structure of the 1.8 Angstrom structure of zinc D-Ala-D-Ala carboxypeptidase [Ghuysen, 2004] shows an alpha/beta-protein with mainly antiparallel beta-sheets. There is a non-catalytic N-terminal module that carries a substrate-recognition and -binding site [Ghuysen et al., 1994]. The structure of the VanX dipeptidase [Bussiere et al., 1998] was reported together with those of complexes with a substrate and with phosphonate and phosphinate transition-state analogue inhibitors. The fold of VanX was similar to those of the *Streptomyces* zinc D-Ala-D-Ala carboxypeptidase and the non-peptidase N-terminal domain of the mouse Sonic hedgehog protein. It has been suggested that an evolutionary relationship exists between the metallopeptidases of family M15 (in clan MD) and those of family M23 containing

lysostaphin in clan MO [Bochtler et al., 2004]. It is proposed that similar active sites contain Zn^{2+} tetrahedrally coordinated by two histidines, an aspartate, and a water molecule with the same core folding motif although in otherwise highly divergent protein folds.

Inhibitors of *Streptomyces* zinc D-Ala-D-Ala carboxypeptidase with thiol, hydroxamate or carboxylate metal-binding functions were described by Charlier et al. (1984). Inhibitors of the VanX dipeptidase have been reviewed by Gao (2002).

4.3 Peptidases of *B. anthracis* and clostridial species

Searches for peptidases similar to proteases of *B. anthracis* (lethal factor) or clostridial species (tetanus toxin, clostridial neurotoxins), not any homologues peptidases in genomes of other bacteria were found. This means that these proteases are species specific. A generic protease inhibitor can therefore not be constructed for the lethal factor or clostridial neurotoxins.

5   Protease inhibitors

5.1   Natural occurring protease inhibitors

5.1.1   *TIMPs*

Tissue inhibitors of metalloproteinases (TIMPs) are the endogenous regulators of the matrix metalloproteinases, the ADAM (a disintegrin and metalloproteinase), and ADAM-TS (ADAMs with thrombospondin repeats) families [Nagase et al., 2006]. There are four mammalian TIMPs identified to date (TIMP-1 to -4), and they are all small molecules of 24 kDa in molecular mass. TIMP inhibits the enzymatic function of MMP by inserting the wedge-shaped edge of its N-terminal domain, the so-called MMP-binding ridge, into the catalytic groove of the target MP to form a tight but essentially noncovalent 1:1 stoichiometric complex. TIMPs inhibit all MMPs tested so far, but TIMP-1 is a poor inhibitor for MT1-MMP, MT3-MMP, MT5-MMP and MMP19. TIMP-3 has shown to inhibit ADAMs (ADAM-10, -12 and -17) and ADAMTSs (ADAMTS-1, -4 and -5). TIMP-1 inhibits ADAM-10. Although TIMP-2 inhibits MMP-2 in high concentrations, it has an important role in activating proMMP-2 in a complex with MT1-MMP.

In addition to MMP-inhibiting activities, TIMPs have many important biological functions. TIMPs can promote or inhibit cell growth, depending on the cell type and inductor [Baker et al., 2002; Visse and Nagasa 2003]. While TIMP-1 and TIMP-2 have anti-apoptotic activity, TIMP-3 is pro-apoptotic. Appendix H gives an overview of common and unique features of TIMPS.

5.1.2   *α_2-macroglobulin*

In mammalian plasma the broad-spectrum proteinase inhibitor α_2-macroglobulin is universally distributed. This plasma protein can inhibit various kinds of bacterial proteases as well as endogenous proteases by physical entrapment of target protease. A complex of α_2-macroglobulin with the protease formed is rapidly taken up into a macrophage [Miyoshi and Shinoda, 2000]. MMP activities in the fluid phase are primarily regulated by α_2-macroglobulin.

5.1.3   *α_1 proteinase inhibitor*

The α_1 proteinase inhibitor, a major plasma proteinase inhibitor, functions primarily as an endogenous inhibitor of neutrophil elastase, a highly tissue-destructive enzyme. This plasma protein is inactivated by various bacterial metalloproteases [Maeda, 1996].

5.1.4   *Serpins*

The serpins make up a superfamily of proteins most of which function as serine proteinases inhibitors. They are single chain proteins, approximately 400 amino acid residues long. Serpins from human plasma have been studied intensively, because they are important regulators of serine proteinases, fibrinolysis and complement activation [Potempa et al., 1994].

5.1.5   *IMPI*

Insects are particularly resistant to micro-organisms. The endogenous defense of insects
is based on cellular and humoral immune response. The IMPI (inhibitor of metallopro-
teinases from insects) isolated from the haemolymph of immunized greater wax moth
(*Galleria mellonella*) larvae represents the first peptidic inhibitor of metalloproteinases
identified in invertebrates. The IMPI is a heat stable, glycosylated, low-molecular-mass
protein and its partially determined amino acid sequence exhibits no similarity to those
of vertebrate TIMPs [Wedde et al., 1998]. Among the metalloproteinases tested, IMPI
was active against several bacterial metalloproteinases of the M4 family of clan MA(E),
the gluzincins. This family of metalloproteinases is known only from bacteria including
human pathogens such as *Legionella, Listeria, Clostridium, Helicobacter,
Pseudomonas* and *Vibrio* [Barrett et al., 1998]. In contrast with the specific inhibition of
gluzincin enzymes, native or rIMPIs exhibit no, or negligible, inhibitory activity against
MMPs of clan MA (M) (metzincins) [Clermont et al., 2004]. As the IMPI inhibits
several microbial metalloproteinases, it has a potential role as a template for
therapeutics against human pathogenic bacteria.

5.1.6   *Other inhibitors*

Other inhibitors are described in Appendix I.

5.2   Synthetic inhibitors

Synthetic inhibitors, small molecule (nonpeptidic) inhibitors, can be identified by
screening the inhibitory properties of compounds. Using a two-stage screening assay,
Panchal et al. (2004) selected six inhibitors of the lethal factor of *Bacillus anthracis* on
the basis of a pharmacophoric relationship determined by X-ray crystallographic data,
molecular docking studies and three-dimensional (3D) database mining. The molecular
scaffolds, including several organometallic and charged molecules, may be used to
develop therapeutically viable inhibitors of LF.
A series of potent 5-amiddino-2-(2-hydroxy-biphenyl-3-yl)-benzimidazoel inhibitors of
plasma kalikrein were identified by Young et al. (2006).
Currently a project is running in which Pyxis Discovery and TNO are cooperating in
finding new small molecule inhibitors to treat anthrax. Pyxis Discovery is focussing on
computational chemistry. In the design of compounds, strict criteria for
pharmacological properties are applied and favourable chemical characteristics for lead
optimization are incorporated to increase the chance of survival in preclinical
development. At TNO, components are screened to inhibit the anthrax lethal factor
using an enzyme- (Quickzyme™ and Smart Man Genomics™) and cell-line-based
assay.
In a study by Burnett et al. (2003), a high throughput assay was used to identify small
molecules that inhibit the metalloprotease activity of botulinum neurotoxins (BoNTs)
serotype A light chain. All inhibitors were further verified using a HPLC-based assay.
Conformational analyse of these compounds, in conjunction with molecular docking
studies, were used to predict structural features that contribute to inhibitor binding and
potency.

5.3   Proteinase inhibitors by *in vitro* evolution

Nature has provided many examples of the co-evolution of peptidases and peptidase inhibitors. For a select number of these systems, structural details of the atomic contacts involved in the interaction between the two protein molecules are known. Unfortunately, for many proteases of medical importance, naturally occurring protein inhibitors have not arisen or have not discovered yet. Therefore, there has been great interest, in adapting known proteinase inhibitors by *in vitro* evolution to do the new job of inhibiting deleterious proteolysis [Dunn and Bungert, 2003].

5.4   Examples of proteases, inhibitors and their clinical status

For various proteases inhibitors have been selected ans tested in clinical trials.
In Table 8, an overview is given [Lüthi, 2002].

Table 8   Proteases, inhibitors and their clinical status.

Protease	Function	Disease	Inhibitor and status
HIV-1 protease	Viral replication	AIDS	Saquinavir (Roche), ritonavir (Abbott), Indinavir (Merck), nelfinavir (Pfizer/agouron), amprenavir (Vertex/Glaxo Wellcome) FDA approved for therapeutic use
Angiotensin-converting enzyme (ACE)	Generation of angiotensin II	Hypertension, congestive heart failure	Captopril, enalapril and enalaprilat, lisinopril, benazepril, moexipril, trandolapril, fosinopril, ramipril, quinapril FDA approved for therapeutic use
Rhinoviru 3C protease	Viral replication	Common cold	Ag7088; Phase II
Proteasome	Protein degradation	Colon, breast, lung cancer	PS-341; Phase II
Tryptase inhibitor	inflammation	Psoriasis, acute inflammatory bowel disease	APC-2059; Phase III
Thrombin	Blood coagulation	Stroke	Ximelagatran; Phase III
ACE + neutral endopeptidase	Generation of angiotensin II	High blood pressure, congestive heart failure	Omapatrilat ACE/NEP inhibitor 100/240; Phase II
ICE (caspase 1)	Conversion of interleukin-1	Rheumatoid arthritis, osteoarthritis	Pralnacasan 3840/VX-740; phase II VX-765; preclinical
Caspases	Apoptosis and inflammation	Sepsis	VX-799; preclinical

Trials may be designed to assess the safety and efficacy of an experimental therapy. Extensive preclinical studies (*in vitro*/animals experiments) are conducted before clinical trials are conducted. These trials are classified into four phases.
- Phase I trials are the first-stage of testing in human subjects.
- Phase II trials are performed on larger groups (20-300) and are designed to assess clinical efficacy of the therapy. A risk assessment evaluates the potential adverse effects that protease inhibitors could have on humans.

- Phase III studies are randomized controlled trials on large patient groups (300-3,000 or more depending upon the condition) and are aimed at being the definitive assessment of the efficacy of the new therapy, in comparison with current 'Gold Standard' treatment.
- Phase IV trials involve the post-launch safety surveillance and ongoing technical support of a drug.

5.5 Inhibitors of plasma kallikrein activity

The following plasma kallikrein inhibitors have been developed to treat hereditary angioedema (HAE) attacks. Hereditary angioedema is an inherited abnormality of the immune system that causes swelling, particularly of the face, and abdominal cramping. The inhibitor is selected for plasma kallikrein [Moreau et al., 2005].

DX88
DX88 is a synthetic kallikrein-inhibitor, based on a recombinant Kunitz-domain (a serine protease inhibitor domain) produced by a phage display technology. *In vivo*, the drug effectively reverses the increased vascular permeability in C1INH-deficient mice at very low intravenous doses. Clinical trials show that the drug was generally well tolerated and improved the clinical symptoms of HAE with the first 4 h following the laryngeal attack.

Aprotinin
Aprotinin is a naturally occurring 58 aminoacid serpin isolated from bovine lung that inhibits serine proteases with a particularly high affinity for plasma kallikrein and plasmin

C1INH
Severe HA-attacks are currently treated by intravenous injection of pasteurized C1INH purified from human blood plasma. Recombinant human C1INH has also been developed and is currently being tested in a clinical trial.

Analog 11
A series of potent 5-amidino-2-(2-hydroxy-biphenyl-3-yl)-benzimidazole inhibitors of plasma kallikrein were identified. Within this series, analog 11 demonstrated the best potency and selectivity profile for plasma kallikrein versus related serine proteases. The pharmacokinetic parameters after *in vivo* dosing to rate indicated that this compound is highly stable *in vivo* and could be further developed for the treatment of inflammatory or coagulation disorder [Young et al., 2006].

6 Summary of possible targets

In this report a selection of human and bacterial peptidases was made that could act as a target for generic peptidase inhibitors. In this chapter, highlights of possible targets will be summarized.

6.1 Trouble with MMP inhibitors

A wealth of knowledge has been accumulated to show that matrixins play many roles in both biological and pathological processes. Biochemical studies of MMPs have characterized their functions and the 3D structures have provided the molecular basis for understanding how these multi-domain proteinases function and interact with ECM molecules and inhibitors. Based on these studies, a large number of MMP inhibitors have been designed and synthesized and some were clinically tested for the treatment of patients. Protease inhibitors that reached clinical trials caused great excitement. HIV protease inhibitors, in combination with reverse transcriptase inhibitors, revolutionized treatment for people with HIV. Other protease inhibitors under development like matrix metalloprotease inhibitors (MMPIs) could stop many types of cancers from spreading throughout the body, but several years down the line, results have failed to live up to expectation [Baker et al., 2002]. The lack of success of early human trials was due to lack of complete knowledge. What once looked simple is now recognized to be a very complex, interdependent system, with the activity of many MMPs being regulated at many different levels, and each MMP being produced and activated in multiple steps and then controlled by endogenous inhibitors. MMPs are entwined in a complex cascade with themselves and other proteases and these proteases could try to compensate if an MMP is blocked. In tumour research it was shown from animal studies, that MMP inhibition can actually stimulate disease progression, possibly by inhibiting beneficial proteases known to slow tumour progression. Challenges in designing selective metalloprotease inhibitors includes not only the identification of the enzymes critical in disease progression, but also the fact that there are more than 50 similar metalloproteases in human and to screen inhibitors for a particular enzyme or set of enzymes.

6.2 Caspases

Other protease inhibitors at the cutting edge of clinical research are the caspase inhibitors. Caspases function in both apoptosis and inflammatory cytokine processing and thereby have a role in resistance to sepsis. In research of Saleh et al. (2006) it was shown that caspase-12 dampened the production of the pro-inflammatory cytokines interleukin IL-1β, IL-18 and IFN-γ, but not tumor-necrosis factor-α and IL-6, in response to various bacterial components that stimulated the TLR pathways. In mice, gene-targeted deletion of caspase-12 renders animals resistant to peritonitis and septic shock. The resulting survival advantage was conferred by the ability of the caspase-12-deficient mice to clear bacterial infection more efficiently than wild-type littermates. It thereby makes it a potentially important target for future therapeutic strategies. However, it was shown by Kachapati et al. (2006) that the gene coding for caspase-12, was previously found only among people of African descent. Therefore, treatments may help strengthen the immune system of those people unfortunate enough to have the caspase-12 gene product.

The current knowledge on programmed cell death primarily comes from investigations on three model organisms, *C. elegans*, *Drosophila* and mammals (see Figure 9). In the mammalian pathway, which has been subject to intense investigations for years, the mechanistic picture remains incomplete. For example, the molecular mechanism by which the apoptosome activates procaspase-9 is not clear. Recent studies of caspase activation, inhibition, and reactivation have galvanized the apoptosis field and will certainly spur more systematic studies on these processes [Shi, 2004].

6.3 Recombinant human activated protein C treatment in sepsis: a drug in trouble

Human APC is an attractive therapeutic for treatment of septic shock. However, the ADDRESS trial showed that the robustness of the data supporting the use of rhAPC in treating patients with severe sepsis may be questioned. Elevated serine protease inhibitor levels could be the reason for the disappointing results. By design and production of derivatives with the goal of altering the proteolytic specificity of APC such that the variants exhibit resistance to inactivation by protein C inhibitor and α_1-antitrypsin yet maintain their primary anticoagulant activity, the therapeutic opportunities for protein C derivatives could be favoured.

6.4 Insect protease inhibitors and *in vitro* evolution

As the IMPI inhibits several microbial metalloproteinases, and no, or negligible, inhibitory activity against MMPs of clan MA (M) (metzincins), it has a potential role as a template for therapeutics against human pathogenic bacteria. In addition to IMPI, a number of novel ISPIs (inducible serine protease inhibitors) have been discovered of immunized *G. mellonella* larva [Clermont et al., 2004]. All three identified ISPIs were determined to inhibit toxic serine proteases produced by the fungus *Metarhizium anisopliae*. The known spectrum of protease inhibitors from invertebrates includes also several well-characterized serine protease inhibitors and one cysteine protease inhibitor [Kanost and Jiang, 1996].

Novel specific (metallo)protease inhibitors can be isolated from insects when the inhibitor production is induced in response to injected microbial elicitors of the innate immune response, such as cell wall components (containing proteases) from micro-organisms or purified proteases.

When the sequence of a cDNA coding for the IMPI is identified, these molecules could be adapted by *in vitro* evolution to improve their catalytic activity or to inhibit other classes/families of proteases. *In vitro* evolution is a new, important laboratory method to evolve molecules with desired properties. It has been used in a variety of biological studies and drug development [Stemmer, 1994]. An adaptation to this method led to a new technique called synthetic DNA shuffling [Ness et al., 2002]. Here, small overlapping synthetic oligonucleotides covering the protein region of interest are generated and assembled into expression clones by PCR. The use of synthetic DNA shuffling has the advantage that desired mutations can be introduced into individual oligonucleotides that then randomly recombine during the assembly step.

6.5 Synthetic protease inhibitors

At the moment, synthetic inhibitors are synthesized and selected against the anthrax lethal factor in a project run by Pyxis Discovery and TNO. Synthetic compounds can also be selected to inhibit other proteases of interest (plasma kallikrein, prepilin peptidase) when new projects are planned.

6.6　　　Bacterial proteases

Bacterial proteases play a significant role in inflammation processes. The bacterial proteases and their inhibition constitute important, emerging research fields both for the drug design of novel therapeutic agents that would avoid drug resistance problems typical of classical antibiotics. Antibiotics available at the moment possess the same mechanism of action, intervening at different points in the bacterial cell wall biosynthesis, explaining the wide-spread cross-resistance to these classical antibiotics. Design of bacterial protease inhibitors would constitute one of the best alternatives to classical antibiotics and may indeed be considered as some of the most important targets for drug design. The aim of this review was to select peptidases that could act as a target for generic peptidase inhibitors in therapy to infectious agents and the MEROPS database was used for this purpose. Homologues sequences of six bacterial peptidases were selected which were found in the A-list and B-list NIAID Priority Pathogens. These sequences were not found in human genome, to rule out that generic peptidase inhibitors affect human proteases. Of these six peptidases, three peptidases are involved in bacterial cell wall synthesis. The other peptidases are involved in repair of single-stranded DNA within the bacterial cell, degradation of soluble and reconstituted fibrillar type I collagen, and one peptidase is involved in type IV pilus formation.

Cell wall construction is necessary for the survival and existence of bacteria but is not a direct virulence factor. For these peptidases (S11, M15 and A8) inhibitors have already been generated and therefore were not selected to be target enzymes. S24 peptidase is involved in SOS response. It is well documented that in *Staphylococcus aureus* and *Escherichia coli*, the SOS response is induced by antibiotics that interfere with cell wall synthesis as well as DNA replication [Courcelle et al., 2001]. Inhibition of the S24 peptidase is only of value when the SOS response is triggered. When bacteria are present in the human body, causing inflammatory processes, it can be assumed that the SOS response is not switched on. The prescription of antibiotics to a person with inflammatory symptoms could lead to triggering of the SOS response. Therefore a combination therapy of antibiotics and inhibitor of the S24 peptidase is more logical than the prescription of a S24 peptidase inhibitor alone.

For synthesis of generic protease inhibitors, the peptidases A24 and U32 are promising targets. U32 is an endopeptidase responsible for the degradation of soluble and reconstituted fibrillar type collagen and therefore directly responsible for tissue damage. However, a problem might be that nothing is known about the active site, and accordingly the peptidase is assigned to a family of unknown catalytic type. More fundamental research is needed to get insight in the catalytic activity of this enzyme.

Pili are important virulence factors for certain types of human bacterial pathogens, such as *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Neisseria gonorrhoeae*. These bacteria all produce type IV pili (Tfp) composed of a single major protein subunit (pilin). The assembly of type IV pili has been most extensively studied in *P. aeruginosa*, in which four assembly proteins (PilB, PilC, PilD and PilQ) have been identified [Bentzmann et al., 2006]. PilD is known to correspond to the prepilin peptidase, which cleaves the pilin precursors, PilA. Intriguingly, several bacteria, not known to have type IV pili, also produce a protein with significant sequence homology to known prepilin peptidase and which is required for extracellular secretion.

Francisella tularensis is a highly infectious Gram-negative bacterium with full potential
for use as a bioweapon. The high infectivity and lethality of aerosolized form have led
to classification of *F. tularensis* as a category A agent of bioterrorism. The molecular
mechanisms that account for the high virulence of *F. tularensis* are largely unknown.
The bacterium makes an unusual lipopolysaccharide. *F. tularensis* expresses a capsule
that protects it against serum-mediated lysis and appears to be necessary for full
virulence. Several genes associated with growth inside macrophages have been
identified. However, apart from the capsule, toxins or other secreted virulence factors
have not been identified. Whole bacterium transmission electron microscopy (TEM)
examination of the live vaccine strain (LVS) of *F. tularensis* revealed the presence of
surface fibers with characteristics of type 4 pili: long, thin, polarly localized structures
that may form bundles of interlinked networks [Gil et al., 2004].
A *Francisella* pathogenicity island encoding several genes that are required for both
intracellular survival in macrophages and virulence in mouse infection model was
recently described. Another gene cluster was found which could play a role in virulence
encoded proteins for secretion and assembly of type IV pili which contained two repeats
of 120 base pairs. Forslund et al. (2006) showed that direct repeat-mediated deletion of
type IV pilin gene results in major virulence attenuation of *F. tularensis* and suggest
that the pilin is essential for infections via peripheral routes. Importantly, the pilin-
negative strain was impaired in its ability to spread from the initial site of infection to
the spleen. Construction/design of a type 4 prepilin peptidase inhibitor could therefore
also result in virulence attenuation. The prepilin peptidase (PilD) is located in the
periplasmic fraction (see Figure 12). Therefore, the selected inhibitor has to be able to
pass the outer membrane of the bacterium in order to reach its target (when the
bacterium is located in a macrophage, also the macrophage membrane has to be
passed).
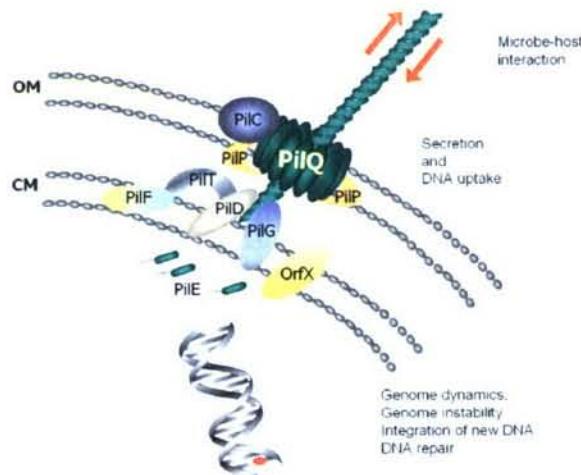
Figure 12   Schematic overview which shows the pili of *Neisseria meningitides*.
             OM: outer membrane; CM: cytoplasmic membrane.

In Table 9 the A and B-listed pathogens are shown having the homologues genome
sequences of the prepilin peptidase. Also a literature reference is included describing
the activity of the prepilin peptidase *in vitro* or/and *in vivo*.

Table 9   A and B-listed pathogens containing the homologues genome sequences of the prepilin
           peptidase (data from Appendix G, AD A24A). A reference is included in which *in vitro* or/and
           in vivo activity of the prepilin peptidase is described.

Bacterium	Reference
V. cholerae	Alam et al. (2005)
B. mallei	Nierman et al. (2004)
B. pseudomallei	Essex-Lopresti et al. (2005)
C. jejuni	Wiesner et al. (2003)
C. burnetii	--
V. paraheamolyticus	Shime-Hattori et al. (2006)
V. vulnificus	Paranjpye and Strom (2005)
L. monocytogenes	--
E.coli O157	Daniel et al. (2006)
S. typhi	Wu et al. (2005)
S. flexneri	--
B. anthracis	--
Y. pestis	Collyn et al. (2002)
F. tularensis	Forslund et al. (2006)

For *B. anthracis* no literature reference was found that showed prepilin activity *in vitro* and/or *in vivo*. Varga et al. (2006) showed that *Clostridium perfringens* was the first example of a Gram-positive bacterium utilizing type IV pili-dependent gliding motility. Also evidence was presented that all nine Clostridium species for which a genome sequence has been determined are capable of type IV pili-dependent motility, including such pathogens as *Clostridium botulinum*, *Clostridium difficile* and *Clostridium tetani*. Sofar, no inhibitor for prepilin peptidase has been described in literature. Selecting and generating a prepilin peptidase inhibitor is therefore an interesting approach to attenuate bacterial virulence.

6.7   Kallikrein/kinin system

Bacteria can use the kallikrein/kinin system for the acquisition of plasma proteins (for growth and proliferation) by deregulating this system which results in an overproduction of bradykinin, a hormone that increases capillary permeability. This was shown in an experiment in which the injection of LPS from *E. coli* into the dorsal skin of rats caused a dose dependent increase in vascular permeability and this increase caused by LPS was attenuated by pre-treatment with the B_2R antagonist HOE 140 [Ueno et al., 1996]. From this experiment the conclusion can be made that when the bradykinin production is stopped, there will be no acquisition of plasma proteins which could be beneficial for bacterial growth and proliferation. Possibilities to modulate the kallikrein/kinin system are shown in Figure 13. To reduce the effects of bradykinine, several pharmacological targets can be modulated:
- prevention of activation of the contact system;
- inhibition of plasma kallikrein activity;
- development and application of B_2R antagonists.

However, modulation of the kallikrein–kinin activity can be a disadvantage for clearance of bacteria after an infection. Frick et al. (2006) showed that in human plasma, activation of the contact system at the surface of significant bacterial pathogens was found to result in further HMWK processing and bacterial killing. A D3 domain of HMWK was generated and within this fragment a sequence of 26 amino acids was

responsible for the antibacterial activity as effectively as the classical human antibacterial peptide LL-37. These data identified a novel role for the contact system in the defence against invasive bacterial infection.

Monteiro et al. (2006) showed that in an infection model (*T. cruzi*; parasite evoked inflammation) the type 1 immunity was vigorously induced by bradykinine, an innate signal whose levels in peripheral tissues are controlled by an intricate interplay of TLR2, B_2R and ACE. This cascade of reactions is shown in Figure 14.
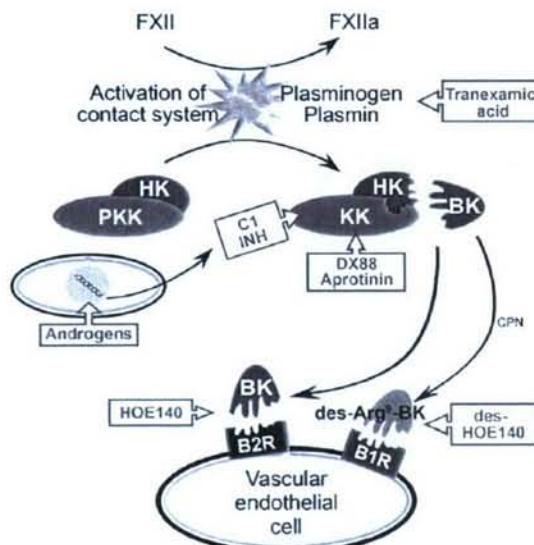
Figure 13   Pharmacological targets to modulate the kallikrein-kinin activity. Tranexamic acid inhibits fibrinolysis and DX88 and C1INH inhibit the serine activity of plasma kallikrein although androgens stimulate the synthesis of C1INH. B_1 and B_2 antagonists block the activation of their receptors [Moreau et al., 2005].
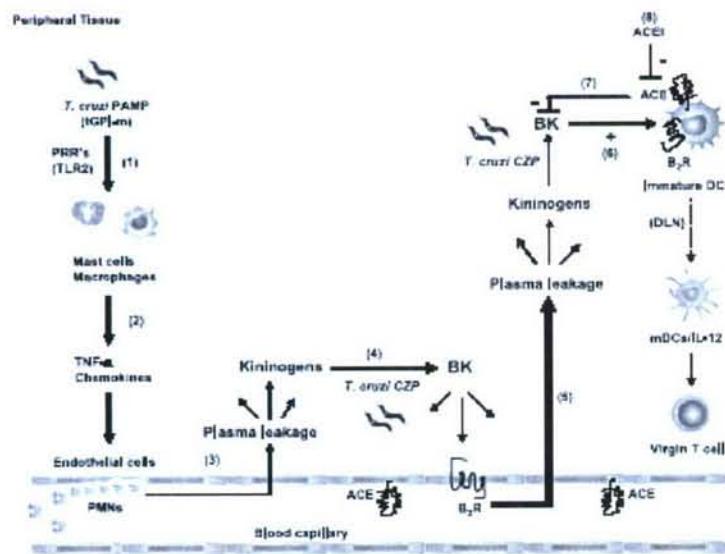
Figure 14   Schematic model depicting the dynamics of parasite-evoked inflammation.

The study of Monteiro revealed that TLR2 activation by microbial signature promotes diffusion of plasma-borne kininogens to the site of infection. PAMPs are recognized by TLR2 expressed by interstitial cells such as macrophages or mast cells ((1) in Figure 14). Activation of these sentinel cells leads to secretion of inflammatory

mediators such as TNF-α and chemokines (2). Circulating PMNs adhere to the activated endothelium. Due to plasma extravasation, plasma-borne kininogens rapidly accumulate in peripheral tissues (3). Acting further downstream, the parasite cysteine protease cruzipain (CZP) processes kininogens, liberating vasoactive kinins (BK) (4). The short-lived kinins activate B_2R of endothelial cells, thus augmenting plasma extravasation (5). As inflammation escalates, the levels of endogenous kinins that are generated in the interstitial tissues is further raised, owing to cruzipain-mediated processing of kininogens. By the liberation of bradykinin, IL-12 production by $CD11c^+$ dendritic cells (DCs) is stimulated and mounts a full-fledged type 1 adaptive response by means of IFN-γ production by T-cells. The innate effects of BK are counter balanced by the kinin degrading activity of angiotensin converting enzyme (ACE) (7). Captopril (inhibitor of ACE; ACEi) promoted increased generation of innate kinin signals in the primary site of infection, thereby stimulating immunity via the B_2R/IL-12 pathway.

Aliberti et al. (2003) showed similar results. They demonstrated that kinins mobilize dendritic cells to produce IL-12 through activation of the B_2-receptor subtype and that bradykinin-induced IL-12 response are tightly regulated by ACE. They concluded that synthetic BK homologues are worth investigating as potential adjuvants in vaccine formulations designed to trigger Th1 responses, either for the development of protective immunity against intracellular pathogens or for the prevention/treatment of allergic disease.

The studies of Aliberti and Monteiro showed an analysis of the molecular mechanisms responsible for the endogeneous generation of kinins in sites of infection. Survival rates of infected animals were not mentioned so no conclusion could be made if this intricate interplay of TLR2, B_2R and ACE results in better infection resistance of the animals. For B_1B_2 deficient mice, the lack of the two receptors stabilized blood pressure in endotoxemia, however, this was not translated into improved survival [Cayla et al., 2007].

Bengtson et al. (2006) showed that at infected sites, invading monocytes became activated by staphylococcal products (toxins) and secrete proinflammatory cytokines that induce an upregulate B_1R at the infectious site (Figure 15). Plasma exudation into the infection site will trigger contact activation and the formation of BK. BK binds to B_2R and trigger its down-regulation or be converted to the B_1R agonist, des Arg^9BK. Induction of B_1R leads to prolonged inflammatory and pain responses. In research of Pesquero et al. (2000) hypoalgesia and altered inflammatory responses (reduced invasion of leukocytes) in mice lacking kinin B_1-receptors were found. They concluded that the kinin B_1 receptor played an essential physiological role in the initiation of inflammatory responses and the modulation of spinal cord plasticity that underlies the central component of pain. Based on the findings of Bengston and Pesquero, a strategy using B_1R antagonist represents a useful approach for the treatment of inflammatory disorder and pain.
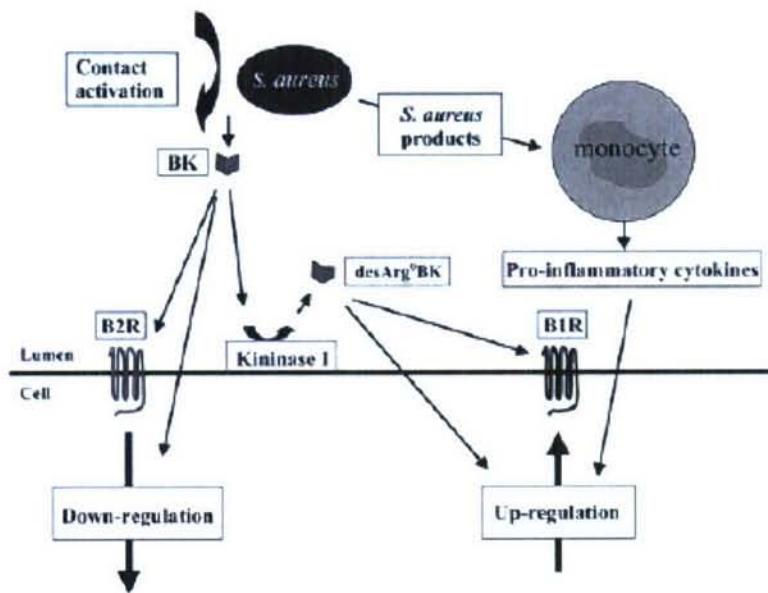
Figure 15   Proposed mechanism used by *S. aureus* to interact with B_1R and B_2R.

7 General conclusions and recommendations for future research

7.1 General conclusions

To prevent or attenuate tissue damaging processes caused by a bacterial infection, proteases have to be identified for which generic protease inhibitors can be selected. In this report, bacteria which belong to the A- and B-list NIAID priority pathogens were screened for the possibility to select generic proteases inhibitors. The prepilin peptidase was selected as a possible target. The selection of a prepilin peptidase was based on homologues genome sequences of the prepilin peptidase. Because the presence of a prepilin peptidase genome does not guarantee the expression of this protein, one can argue about its function in virulence. A good example is the genomic presence of prepilin peptidase in *B. anthracis*. Sofar, only for the Gram-positive bacterium *Clostridium perfringens* type IV pili-dependent gliding motility was shown.

Future research has to prove if the prepilin peptidase of *B. anthracis* (and other bacteria from which only the homologues prepilin peptidase gene sequence is known) has a function in its virulence. Thereby, the inhibitor of the prepilin peptidase has to cross the bacterial membrane because the prepilin peptidase is located in the membrane with a cytoplasmic orientation. When the infection is caused by an intracellular bacterium, also the macrophage membrane has to be passed. For the delivery of the inhibitor to its action site, some kind of shuttle system has to be applied. For the synthesis and delivery of an effective prepilin peptidase inhibitor, these fundamental questions have to be resolved first.

A disadvantage of selecting a bacterial protease inhibitor is that inflammation caused by other biowarfare agents like viruses or toxins can not be treated. It would be more appropriate to select human proteases which have a function in the coordinated response aimed at the protection of the host at the onset of an inflammatory response. In this report the kallikrein/kinin system has been described as a cascade pathway by which the inflammatory response can be modulated. In Paragraph 6.7 (Figure 13) it was shown that there are several pharmalogical targets to modulate the kallikrein-kinin activity. The conclusion was made that synthetic BK homologues are worth investigating as potential adjuvants in vaccine formulations designed to trigger Th1 responses for the development of protective immunity against intracellular pathogens. The type 1 immunity was also vigorously induced by bradykinine, an innate signal whose levels in peripheral tissues are controlled by an intricate interplay of TLR2, B_2R and ACE. Next to the B_2R, a strategy using B_1R antagonist represents a useful approach for the treatment of inflammatory disorder and pain. The kallikrein-kinin system can modulate both the innate and adaptive immunity and could therefore represent a promising approach for the development of novel strategies to treat bacterial infections.

7.2 Recommendations for future research

An inappropriate host response to invading bacteria is a critical parameter that often influences the outcome of an infection. Cytokines are instrumental in cell-mediated immunity (Th1 response, proinflammatory response responsible for killing intracellular parasites) against a broad spectrum of pathogens. A shift in the balance between pro- and anti-inflammatory cytokines results in impaired defence against microbial pathogens.

The kallikrein-kinin system can modulate both innate and adaptive immunity and could therefore represent a promising approach for the development of novel therapies for treatment of bacterial infections. Recently, gene-targeting technologies generated genetically altered animal models in the kallikrein-kinin system: mice deficient in B_1, B_2, and B_1/B_2 kinin receptor. The use of these genetically altered animal models can lead to *in vivo* insights into the role of the kallikrein-kinin system in inflammatory processes. Today, at TNO Defensie, Security and Safety, location Rijswijk, a pulmonary infection model (mice) is used with *Listeria monocytogenes* as infection agent. With this model, various compounds are tested for their capability to boost the immune response. The experience gained in these experiments can be used in the research in which transgenic mice are used. Also here, a pulmonary infection model with *L. monocytogenes* can be applied to get insight in the role the kallikrein-kinin system.

Several clinical trials targeting single systems to treat septic shock (inhibition of kinins, TNF-α, prostaglandins) have yielded very little positive results. Tackling a single system does not seem to be successful in a complex and multifactorial inflammatory disease [Cayla et al., 2007]. Future research in which the blockade of kinin receptors only or in combination with other compounds (see for example the research which showed that an intricate interplay of TLR2, B_2R and ACE resulted in the induction of the type 1 immunity [Monteiro et al., 2006]), might result in the development of treatment to protect the host at the onset of infection. With *in vivo* models the survival rate of infected animals can be determined.

8 Literature

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9 Signature

Rijswijk, August 2007

TNO Defence, Security and Safety
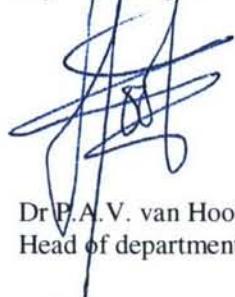
Dr P.A.V. van Hooft
Head of department

Dr A.F.W.M. Wolterink, MSc
Author

Dr J. Kieboom
Author

A Content of granula of neutrophils

Serine Proteases
Elastase
Cathepsin-G
Proteinase 3
P29b (=AGP7)

Hydrolases
β-glucuronidase
β-glycerophosphatase
N-acetyl-β-glucosaminidase
A-mannosidase
Cathepsin-B
Cathepsin-D

Metallo-proteases
Collagenase
Gelatinase

Other proteins
Myeloperoxydase
Tetranectine
Lysozyme
Histaminase
Albumin
Azurocidin
Lactoferrin
Defensin
Vitamin B12-binding protein
Bactericidal permeability-increasing protein
γ-glutamyltransferase
Adenosine-diphosphatase
Neutral α-glucosidase
aminopeptidase N (CD13)
LFA-1 (CD11a/CD18)
CD3 (CD11b/CD18)
P150,95 (CD11c/CD18)
Tyrosine phosphatase (CD45)

B   Vertebrate MMPs and substrates

Name	Common names	Activators	Inducers	Some substrates
MMP-1	Collagenase-1, interstitial-collagenase	Plasmin, MMP-3, -7 and -10	TNFα, IL-1β, PDGF, EGF	Coll III > I > II, VII, VIII, Gel, En, Tn, Per, Lm, Ag, Cas, proTNF-α, proIL-1β, IL-1β, IGF-BP, proMMP-1 and -2, α1-PI, α1-ACT, α2-MG, MCPs
MMP-2	Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase	MT-MMP, MMP-1 and -13	TGFβ	Gel, Coll I, III, IV, V, VII, X, XI, El, Fn, Lm, AG, Vn, Dc, PI, proTGF-β1, proTNF-α, proIL-1β, IGF-BP, FGF-R1, proMMP-1, -2 and -13, α1-PI, α2-MG, MCP-3, On
MMP-3	Stromelysin-1, transin-1	Plasmin	TNFα, IL-1β, EGF	Ag, Ln, Fn, Coll III, Iv, V, IX, X, XI, XVIII, Gel, Dc, En, Per, Tn, Vn, Fb, El, Lm, Cas, pro-TNF-α, pro-HB-EGF, proIL-1β, Per, PI, E-cadherin, IGF-BP, pro-MMP-1, -3, -7, -8, -9 and -13, α1-PI, α2-MG, MCP-3, On, α1-ACT
MMP-7	Matrilysin, PUMP-1	Plasmin	LPS	Fn, Lm, Coll I, IV, V, IX, X, XI, XVIII, Gel, Ag, En, Tn, Vn, Dc, Fb, Cell surface FasL, pro TNF-α, E-cadherin, β4 integrin, PI, pro-MMP-1, -2, -7 and -9, α1-PI, α2-MG, On
MMP-8	Collagenase-2, neutrophil collagenase	MMP-3, -7 and -10	TNFα, IL-1β	Coll I > II > III, VII, X, Gel, En, Ag, Tn, proTNF-α, IGF-BP, proMMP-8, α1-PI, α2-MG, MCP-1
MMP-9	Gelatinase B, 92-kDa gelatinase	Plasmin, MMP-3, -13 and 26	TGFβ, TNFα, IL-1β, LPS, EGF	Gel, Coll I, IV, V, VII, X, XI, XIII, El, Dc, Fn, Lm, Ag, Vn, Cas, proIL-8, PF-4, proTGF-β1, proTNF-α, pro-IL-1β, FGF-R1, PI, proMMP-2, -9 and -13, α1-PI, α2-MG, ICAM-1, On
MMP-10	Stromelysin-2, transin-2	Plasmin	TNFα, EGF, TNFα	Coll I, III, IV, V, Gel, El, Cas, Fn, Lm, Pgl, proMMP-1, -8 and -10
MMP-11	Stromelysin-3	Furin	TGFβ, EGF, IL-6, PDGF	Fn, Lm, Ag, IGF-BP, α1-PI, α2-MG
MMP-12	Metalloelastase, macro-phage elastase		TNFα, IL-1β, PDGF	El, Coll I, IV, Fn, Lm, Pgl, Fb, PI, proTNF-α, α1-PI
MMP-13	Collagenase-3	Plasmin, MT-MMP, MMP-2, -3 and -10	LIF, TNFα, IL-1β	CollII > III > I, VII, X, XVIII, Gel, En, Tn, Ag, Lm, proTNF-α, proMMP-9 and 13, α1-ACT, α2-MG, MCP-3
MMP-14	MT1-MMP		TNFα, IL-1β, EGF	Coll I, II, III, Gel, Fn, Lm, Vn, Ag, Cell surface CD44 and tTG, proTNF-α, proMMP-2 and -13, pro αv integrin, α1-PI, α2-MG, MCP-3

Appendix B (continued).

Name	Common names	Activators	Inducers	Some substrates
MMP-15	MT2-MMP		Stretch	Pgl, proMMP-2, Cell surface tTG, proTNF-α
MMP-16	MT3-MMP			Coll III, Fn, proMMP-2, Cell surface tTG, proTNF-α
MMP-17	MT4-MMP			Gel, Fb, proMMP-2, proTNF-α
MMP-18	Collagenase-4			Coll I, II, III, Gel
MMP-19	RASI, stromelysin-4		Phorbol ester	Coll I, IV, Gel, Lm, Fn, Tn, En, AG, Fb, Cas
MMP-20	Enamelysin	MT1-MMP		Amg, Ag, Coll XVIII, Lm
MMP-21	Xenupus MMP			Gel
MMP-22	Chicken homolog of MMP-27			
MMP-23	Cysteine aaray (CA) MMP			Gel
MMP-24	MT5-MMP			Fn, Pgl, Gel, proMMP-2
MMP-25	MT6-MMP		IL-8	Coll IV, Gel, Fn, Pgl, Ln-1, Fb, pro-MMP-2 and -9, α1-PI
MMP-26	Matrilysin-2, endometase			Coll IV, Gel, Fn, Fb, pro-MMP-9, α1-PI, IGF-BP
MMP-27				
MMP-28	Epilysin	furin		Cas

Sternlicht and Werb (2001); Overall (2002); Visse and Nagase 2003; Pirilä et al. 2003.
List of abbreviations can be found in the chapter preceding Chapter 1.

C   Biological activities mediated by MMP cleavage

Biological effect	Responsible MMPs	Substrate cleaved
Keratinocyte migration and reepithelialization	MMP-1	Type I collagen
Osteoclast activation	MMP-13	Type I collagen
Neurite outgrowth	MMP-2	Chondroitinsulphate proteoglycan
Adipocyte differentiation	MMP-7	Fibrinogen
Cell migration	MMP-1,-2, and -3	Fibrinogen
Cell migration	MT1-MMP	CD44
Mammary epithelial cell apoptosis	MMP-3	Basement membrane
Mammary epithelial alveolar formation	MMP-3	Basement membrane
Epithelial-mesenchymal conversion (mammary epithelial cells	MMP-3	E-cadherin
Mesenchymal cell differentiation with inflammatory phenotype	MMP-2	Not identified
Platelet aggregation	MMP-1	Not identified
Generation of angiostatin-like fragment	MMP-3, -7, -9, -12	Plasminogen
Generation of endostatin-like fragment	MMPs	Type XVIII collagen
Enhanced collagen affinity	MMP-2, -3, -7, -9, -13	BM-40 (SPARC/Osteonectin)
Kidney tubulogenesis	MT1-MMP	Type I collagen
Release of bFGF	MMP-3, -13	Perlecan
Increased bioavailability of IGF1 and cell proliferation	MMP-1, -2, -3, -7, -19	IGFBP-3
	MMPs	IGFBP-5
	MMP-11	IGFBP-1
Activation of VEGF	MMPs	CTGF
Epithelial cell migration	MMP-2, MT1-MMP, MMP-19	Laminin 5γ2 chain
	MT1-MMP	Lamin 5β3
Apoptosis (amnion epithelial cells)	Collagenase	Type I collagen
Pro-inflammatory	MMP-1, -3, -9	Processing IL-1β from the preursor
Tumor cell resistance	MMP-9	ICAM-1
Anti-inflammatory	MMP-1, -2, -9	IL-1β degradation
Anti-inflammatory	MMP-1, -2, -3, -1, -14	Monocyte chemoatractant protein-3
Increased bioavailability of TGF-β	MMP-2, -3, -7	Decorin
Disrupted cell aggregation and increase cell invasion	MMP-3,MMP-7	E-cadherin
Reduced cell adhesion and spreading	MT1-MMP, MT2-MMP, MT3-MMP	Cell surface tissue transglutaminase
Fas-receptor mediated apoptosis	MMP-7	Fas ligand
Pro-inflammatory	MMP-7	Pro-TNFα

Appendix C (continued).

Biological effect	Responsible MMPs	Substrate cleaved
Osteocleast activation	MMP-7	RANK-ligand
Reduced IL-2 response	MMP-9	IL-2Rα
PAR1 activation	MMP-1	Protease activated receptor 1
Generation of vasoconstrictor	MMP-2	Big endothelin
Conversion of vasodilator to vasoconstrictor	MMP-2	Adrenomedullin
Vasocontriction and cell growth	MMP-7	Heparin-binding EGF
Neuronal apoptosis leading to neurodegeneration	MMP-2	Stromal cell-derived factor 1α (SDF-1)
Bioavailability of TGFβ	MMP-9	Precursor of TGFβ
Thymic neovascularization	MMP-9	Collagen IV
Hypertrophic chondrocytes apoptosis and recruitment of osteoclast	MMP-9	Galactin-3
Embryo attachment to uterine epihelia	MT1-MMP	MUC1, a transmembrane mucin

D   Characteristics of bacterial toxins

Organism/toxin	Mode of action	Target	Disease
Damage membranes			
Aeromonas hydrophila/ aerolysin	Pore-former	Glycophorin	Diarrhea
Clostridium perfringens/ perfringolysin O	Pore-former	Cholesterol	Gas gangrene
Escherichia coli/ hemolysin	Pore-former	Plasma membrane	UTIs
Listeria monocytogenes/ listeriolysin O	Pore-former	Cholestorol	Illness meningitis
Staphylococcus aureus/ A-toxin	Pore former	Plasma membrane	Abcesses
Streptococcus pneumoniae/ pneumolysin	Pore-former	Cholesterol	Pneumonia
Streptococcus pyogenes/ streptolysin O	Pore-former	Cholesterol	Strep throat
Inhibit protein synthesis			
Corynebacterium diphtheria/diphtheria toxin	ADP- ribosyltransferase	Elongation factor 2	Diphtheria
E. coli; Shigella dysenteriae/shiga toxin	N-glycosidase	28S rRNA	HUS
Pseudomonas aeruginosa/ exotoxin A	ADP- ribosyltransferase	Elongation factor 2	Pneumonia
Activate second messenger pathways			
E.coli/CNF	Deamidase	RhoG-proteins G-proteins	UTIs
LT	ADP- ribosyltransferase	Guanylate	Diarrhea
ST	Stimulates guanylate cyclase	cyclase receptor	Diarrhea
Bacillus anthracis/edema factor	Adenylate cyclase	ATP	Anthrax
Bordetella pertussis/ dermonecrotic toxin	Deamidase	Rho G proteins G-proteins	Rhinitis
pertussis toxin	ADP- ribosyltransferase		Pertussis
Clostridium botulinum/ C2 toxin	ADP- ribosyltransferase	Monomeric G-actin	Botulism
C3 toxin	ADP- ribosyltransferase	Rho G-protein	Botulism
Vibrio cholerae/ cholera toxin	ADP- ribosyltransferase	G-proteins	Cholera

Appendix D (continued).

Organism/toxin	Mode of action	Target	Disease
Clostridium difficile/ toxin A	Glucosyltransferase	Rho G-proteins Rho G-proteins	Diarrhea
toxin B	Glucosyltransferase		Diarrhea
Activate immune respons			
S. aureus/ Enterotoxins	Superantigen	TCR/MHC II	Food poisoning
exfoliative toxins	Superantigen and serine protease		SSS
toxic-shock toxin	Superantigen		TSS
S. pyogenes/ pyrogenic exotoxins	Superantigens	TCR/MHC II	SF/TSS
Proteases			
B. anthracis/ lethal factor	Metalloprotease	MAPKK1/ MAPKK	Anthrax
C. botulinum/ neurotoxins A-G	Zinc-metalloprotease	VAMP/synapto brevin	Botulism
Clostridium tetani/ tetanus toxin	Zinc-metalloprotease	VAMP/synapto brevin	Tetanus

Schmitt et al. (1999).

E Bacterial serine and cysteine proteases

Protease	Family	Organism(s)
Streptomyces trypsins	S1	*Streptomyces spp.*
Streptogrisin B	S2	*Streptomyces spp.*
Streptogrisin A	S2	*Streptomyces spp.*
Glutamyl endopeptidase I	S2	*Bacillus subtilis, Staphylococcus aureus*
Glutamyl endopeptidase II	S2	*Streptomyces spp.*
Exfoliative toxin A	S2	*Staphylococcus aereus*
A-Lytic protease	S2	*Achromobacter lyticus, Lysobacter enzymogenes*
DegP (protease Do)	S2	*Bacillus subtillis, Brucella abortus, Campylobacter jejuni, Chlamydia trachomatis, Escherichia coli, Mycobacterium leper, M. paratuberculosis, Rickettsia typhi, R. tsutsugamushi, Salmonella typhimurium, Yersinia enterocolitica*
Lysyl endopeptidase	S2	*Achromobacter lyticus*
IgA1-specific serine endopeptidase	S6	*Haemophilus influenzae, Neisseria gonorrhoeae, N. meningitidis*
C5a peptidase	S8	*Streptococcus agalactiae, S. pyogenes*
Dichelobacter basic serine proteinase	S8	*Dichelobacter nodosus*
Trepolisin	S8	*Treponema denticola*
Tripeptidyl-peptidases	S8, S33	*Streptomyces spp.*
Prolyl tripeptidyl-peptidase	S9	*Porphyromonas gingivalis*
Prolyl aminopeptidase	S33	*Flavobacterium meningosepticum, Mycoplasma genitalium, E. coli, N. gonorrhoeae*
Streptomyces K15 D-Ala-D-Ala transpeptidase	S11	*Streptomyces K15*
Streptomyces R61 D-Ala-D-Ala carboxypeptidase	S12	*Streptomyces spp.*
Signal peptidase I	S26	*E. coli, H. influenzae, M. tuberculosis, Pseudomoans fluorescens, S. typhimurium, Streptococcus pneumoniae*
Omptin	S18	*E. coli, Yersinia pestis, Salmonella typhimurium*
Endopeptidase Clp	S14	*E.coli, B. subtilis, Listeria spp*
Streptopain	C10	*Porphyromonas gingivalis, Streptococcus pyogenes*
Clostripain	C11	*Clostridium histolyticum*
Bacterial pyroglutamyl-peptidase	C15	*Pseudomonas fluorescens, Staphylococcus aureus, S. pyogenes*
Gingipain R, Gingipain K	C25	*Porphyromonas gingivalis*
Sortase	?	Ubiquitous in gram-positive bacteria

F   Bacterial Metalloproteases

Protease	Family	Organism(s)
Listeria metalloprotease	M4	*Listeria monocytogenes*
Coccolysin	M4	*Enterococcus faecalis*
Hemagglutinin/protease	M4	*Vibrio cholerae, Helicobacter pylori*
Pseudolysin	M4	*Pseudomonas aeruginosa*
Legionella metalloendopeptidase	M4	*Legionella pneumophila*
Vibrio collagenase	M9	*Vibrio alginolyticus, V. parahaemolyticus*
Clostridium collagenases	M9	*Clostridium histolyticum, C. perfringens*
Non-haemolytic enterotoxin	M9	*Bacillus cereus*
Serralysin	M10	*Serratia spp., Pseudomonas aeruginosa, Erwinia chrysanthemi*
Aeruginolysin	M10	*Pseudomonas aeruginosa*
Mirabilysin	M10	*Proteus mirabilis*
Fragilysin	M10	*Bacteroides fragilis*
Flavastacin	M12	*Flavobacterium meningosepticum*
Carboxypeptidase T	M14	*Streptomyces spp.*
Leucyl aminopeptidase	M17	*Chlamydia trachomatis, E.coli, Haemophilus influenzae, Mycobacterium tuberculosis, Mycoplasma genitalium, Rickettsia prowazekii, S. typhimurium*
Methionyl aminopeptidase I	M24	*Bacillus spp., Clostridium perfringens, E.coli, Haemophilus influenzae, Klebsiella pneumoniaea, Mycoplasma spp, Salmonella typhimurium, Synechocystis spp.*
Glutamate carboxypeptidase	M20	*Pseudomonas spp., Flavobacterium spp., Acinetobacter spp.*
VanX D,D-dipeptidase	M19	*Enterococcus spp., Synechocystis spp.*
O-Sialoglycoprotein endopeptidase	M22	*H. influenzae, M. leprae, Mycoplasma genitalium, Pateurella haemolytica*
B-Lytic metalloendopeptidase	M23	*Lysobacter enzymogenes*
Staphylolysin	M23	*Aeromonas hydrophila, Pseudomonas aeruginosa*
IgA-specific metalloendopeptidase	M26	*Streptococcus spp., Neisseria spp, Haemophilus spp., Ureaplasma spp., Clostridium spp., Capnocytophaga spp., Prevotella spp.*
Tentoxilysin	M27	*Clostridium tetani*
Bontoxilysin	M27	*Clostridium botulinum, C. barati, C. butyricum*
Anthrax toxin lethal factor	M34	*Bacillus anthracis*
Lysostaphin	M37	*Staphylococcus staphylolyticus, S. simulans*
Aureolysin	M4	*Staphylococcus aureus*
AAA protease	M41	*ubiquitous*

G Presence or absence of selected proteases in B-list pathogens

	AC A8	SE S11	SF S24	U- U32	AD A24A	MD M15
B. melitensis	1	1	1	1	0	1
V. cholerae	1	1	1	1	1	1
B. abortus	1	1	1	1	0	0
B. suis	1	1	1	1	0	1
R. prowazekii	1	1	0	0	0	0
B. mallei	1	1	1	1	1	1
B. pseudomallei	1	1	1	1	1	1
C. jejuni	1	0	1	1	1	0
C. burnetii	1	1	1	0	1	0
V. paraheamolyticus	1	1	1	1	1	1
V. vulnificus	1	1	1	1	1	1
L. monocytogenes	1	1	1	1	1	1
E.coli O157	1	1	1	1	1	1
S. typhi	1	1	1	1	1	1
S. flexneri	1	1	1	1	1	1

0: Species does not contain homologues sequence of protease.
1: Species contains homologues sequence of protease.

H   Common and unique features of TIMPs

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Protein kDa	28	21	24/27	22
N-glycolysation sites	2	0	1	0
Protein localization	Soluble	Soluble/cell surface	ECM	Soluble/cell surface
Pro-MMP association	Pro-MMP-9	Pro-MMP-2	Pro-MMP-2/-9	Pro-MMP-2
MMPs poorly inhibited	(MT1, 2, 3, 5)-MMP, MMP-19	none	none	none
ADAM inhibition	ADAM 10	none	ADAM 12, 17, 19, ADAMTS-4, -5	none
Cell proliferation	↑ Erythroid precursors ↑ Tumour cells	↑ Erythroid Precursors ↑ Tumour cells ↑ Fibroblast ↑ Smooth muscle cells ↓ Endothelial cells	↑ Smooth muscle cells and cancer cells	↑ Mammary tumour cells ↓ Wilm's tumour cells
Apoptosis	↓ Burkitt's lymphoma cells	↑ Colorectal cancer cells ↓ Melanoma	↑ Smooth muscle cells ↑ Tumour cells ↑ retinal pigmented epithelial cells ↓ Melanoma	↑ Cardiac fibroblasts

(Baker et al., 2002).

I   Protease inhibitors

Protease inhibitor	Description/specification of inhibitor
AEBSF-HSL	Irreversible inhibitor of thrombin and other serine proteases. Inhibits by acylation of the active site of the enzyme. Much less toxic than PMSF and DFP
EDTA-Na$_2$	Reversible inhibitor of metalloproteases
EGTA	Inhibits metalloproteases. Reveals high selectivity for Ca^{2+} over Mg^{2+}ions
Phosphoramidon	Specific inhibitor of thermolysin and neutral endopeptidase-24.22. Inhibits also the activity of Endothelin converting enzyme, collagenase and metallo-endoproteinases from many micoorganisms. Does not inhibit serine, cysteine and aspartic proteases
Antipaindihydro-chloride	Papain, trypsin, cathepsin B
Aprotinin	Serine proteases
Bestatin	Aminopeptidases
Chymostatin	Chymotrypsin
E-64	Thiol proteases
Pepstatin	Acid proteases
Pefabloc SC	Serine proteases
Leupeptin	Serine and thiol proteases

J   Activity of selected proteases

Peptidase family	Function
M24	The methionyl aminopeptidases of subfamily M24A are essential for the removal of the initiating methionine of many proteins, acting co-translationally in association with the ribosomes. The X-Pro dipeptidase found in eukaryotes has a role in the cleavage of Xaa Pro linkages found in dipeptides associated with collagen recycling. Deficiency results in an increase of these dipeptides to toxic levels.
M20	In general, the peptidases hydrolyse the late products of protein degradation so as to complete the conversion of proteins to free amino acids. The *Pseudomonas* glutamate carboxypeptidase is a periplasmic enzyme that is synthesised with a signal peptide. Gly-X carboxypeptidase is a yeast enzyme that has a vacuolar localisation, and is synthesised with an N-terminal propeptide. In the cytosolic fractions of mammalian cells there are the non-specific dipeptidase and carnosinase.
M22	O-Sialoglycoprotein endopeptidase has been shown to cleave glycophorin A and the leukocyte surface antigens CD34, CD43, CD44 and CD45. All of these substrates are heavily O-sialoglycosylated, and removal of the carbohydrate prevents cleavage. *Pasteurella haemolytica* is the causative agent of cattle shipping fever, but as yet O-sialoglycoprotein endopeptidase has not been demonstrated to have any involvement in the disease. O-Sialoglycoprotein endopeptidase does not possess a signal peptide and is presumably not secreted.
M50	These peptidases are involved in the regulation of gene expression by proteolysis of transcription regulators. S2P peptidase, in the mammalian endoplasmic reticulum membrane, releases the N-terminal transcription factor domain from membrane-bound SREBPs, and the liberated domain then enters the nucleus and activates genes that control cholesterol uptake and synthesis. Sporulation factor SpoIVFB from *Bacillus subtilis* is involved in the later stages of the sporulation process, in which a peptidoglycan layer surrounds the protoplast.
T1	The proteasome is involved in the turnover of intracellular proteins, including proteins specifically targeted for degradation by polyubiquitination.
S26	The use of signal peptides to direct newly synthesised proteins to a secretory pathway is common to all three domains of living organisms, and the peptidases of family S26 are the major enzymes that remove the signal peptides and facilitate secretion
S16	The function of Lon peptidase in bacteria is thought to be the degradation of unfolded proteins. One physiological substrate of Lon peptidase in *E. coli* is SulA protein, which is a particularly unstable protein that is a cell-division inhibitor.

K Pharmacological and clinical application of kinin B_1-receptor ligand

Ligands Agonist	Application	Studies
R-838 (Sar-[d-Phe^8]des-Arg^9-BK)	Metabolically stable	Rabbit
	High affinity and selectivity Hypertension Stimulation of vasculature formation (following ischemia)	Rodent
Ligands Antagonists	**Application**	**Studies**
[Leu^8]des-Arg^9-BK	Pain	Rat Mice
	Ischemic vascular disease	Mice
Lys-[Leu^8]des-Arg^9-BK	Optimal B_1-receptor antagonist	Human B_1-receptor
Ac-Lys-[MeAla^6,Leu^8] des-Arg^9-BK	Metabolically stable (not very potent compared with the affinity of the reference compound Lys-[Leu^8]des-Arg^9-BK	Rabbit
R-175(Ac-Lys[βD-Nal^7,Ile^8] des-Arg^9-BK)	High affinity Allergic lung inflammation	Human and rabbit B_1-receptor Mice
B9858(Lys-Lys-[Hyp^3, Igl^5, D-Igl^7, Oic^8] des-Arg^9-BK)	Fairly high selectivity for B_1-receptor due to Lys^0 Metabolically stable residue	Human and rabbit B_1-receptor
Des-Arg^{10}-HOE 140	Residual antagonistic effects on B_2-receptor Moderate affinity	Rabbit jugular vein, guinea pig ileum, rabbit aorta
B9430(D-Arg-[Hyp^3, Igl^5, D-Igl^7, Oic^8]-BK)	Mixed B_1-receptor and B_2-receptor antagonist even if desArg^9 fragments has substantial selectivity for B_1-receptor	Demonstration of compatibility of B_1-receptor and B_2-receptor structure by the accommodation of a single antagonist pharmacophore
R-954(Ac-Orn-[Oic^2, α-MePhe^5, D-βNal^7, Ile^8] des-Arg^9-BK)	Allergic lung inflammation Airwy allergy	Mice Rat model, speculative on human
PS020990	Potent and competitive B_1-receptor antagonist High affinity	Human receptor (no *in vivo* data)
Compound 12 (benzodiazepine-based structure)	SSelective antagonist	Human and rat B_1-receptor *in vitro* (equal activity)
Benzo-sulfonylamide compounds Compound 12 Compound 11 SSR240612	Poweful and selective antagonists Hyperalgesia Speculatiev on pain, inflammation and sepsis Inflammation and hyperalgesia	Rat, dog, orally bioavailable Rabbit aortic preparations, rabbit jugular vein Mice, rat, oral activity

Moreau et al., 2005.

L   Pharmacological and clinical application of kinin B_2-receptor ligand

Ligands agonists	Application	Studies
Labradimil ([Hyp^3, Thi^5, 4-Me-Tyr^8 Ψ (CH$_2$-NH) Arg^9]-BK	Vascular permeability (blood brain barrier): adjuvant to chemotherapy of brain tumors)	*In vivo* rodent models Human: phase II studies on glioma
FR190997	Hypertension	Rat
Ligands antagonists	Application	Studies
[D-Phe^7]-BK	Low potency, Antagonist/partial agonist actiity	Rat uterus, guinea pig ileum Rat
[Thi^{5,8}, D-Phe^7]-BK	Potent antagonist, no agonist activity	Rat uterus, guinea pig ileum
HOE 140 (Icatibant; D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK	High affinity, long-lasing, competitive activity in but measurable affinity for B_1-receptor No residual agonist effects Resistance to peptidases Acute rhinitis Astma Early stage of inflammation Persistent inflammatory pain	Animal models (high affinity for human, rbbit, and guiea pig B_2-receptor) Human, nasal treatment Human Rat
Phosphonium family		
WIN64338	Inactive Limited affinity	On human tissues For guinea pig B_2-receptor
WIN62318	Micromolar binding affinity to human B_2-receptor	Identifiation of the absolute requirement for B_2-receptor binding affinity: presence of two positive charges at a distance of about 10Å separated by a lipophilic residue, playing the role of Phe^8 side chain in the native ligand
Quinoline and imidazol [1,2-α]pyridine family	High B_2-receptor affinity and selectivity versus B_1-receptor	Oral activity at doses ranging between 1 and 30 mg/kg in different tests and species
FR165649, FR173657, FR184280	Oral activity on hyperalgesia and inflammation	Rat, mice
FR167344	Selective and high potent binding activity Bronchoconstricition	Guinea pig ileum, human A-431 cells Guinea pigs (oral activity) Designed as clinical candidate to treat inflammatory diseases
Compound 38	High affinity	Human B_2-receptor

Appendix L (continued).

Ligands agonists	Application	Studies
CP2522	High affinity Modeled on CP0597 by replacing β-turn conformation of the peptide by a rigid 1,4-piperazine ring	Human B_2-receptor
Substituted 1,4-dihydropyridines	B_2-receptor antagonist at the nanomolar range	Human B_2-receptor
Bradyzide	Hypertension inflammation	Rodent, orally active, less potent in human B_2-receptor
Natural compounds		
Pyrroloquinoline alkaloid: Martinelline	Affinity for both B_1-receptor and B_2-receptor at the micromolar range but not selective	Alkaloid isolated from the South American tropical plant *Martinella iquitosensis*
L-755807	Inhibition of BK binding to cloned human B_2-receptor at micromolar range	Complex metabolite isolated from a culture of the mould Microsphaeropsis sp. No further pharmacological data

Moreau et al., 2005.

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15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

Inflammation is a coordinated response aimed at the protection of the host at the onset of infection. In this process, human and bacterial proteases play a critical role. The regulation of human proteases is carried out by pro- and anti-inflammatory signals. Deregulation of human (metallo)peptidases results in many pathological reactions such as microbial invasion or inflammatory tissue damage. Also bacterial proteases can influence the balance between pro- and anti-inflammatory signals and thus cause tissue damage. A disadvantage of selecting a bacterial protease inhibitor is that inflammation caused by other biowarfare agents like viruses or toxins can not be treated. It would be more appropriate to select human proteases which have a function in the coordinated response aimed at the protection of the host at the onset of an inflammatory response. The kallikrein-kinin system can modulate both the innate and adaptive immunity and could therefore represent a promising approach for the development of novel strategies to treat bacterial infections. Efforts in future research in which the blockade of kinin receptors only or in combination with other compounds might result in the development of treatment to protect the host at the onset of infection.

16. DESCRIPTORS		IDENTIFIERS
Proteases	Kallikrein-kinin system	-
Protease inhibitors	Inflammatory processes	
MEROPS	Innate and adaptive immunity	

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